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THE BIOLOGICAL BULLETIN

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INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except those written at the specific invitation of the Editorial Board), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the board.

1. *Manuscripts.* Manuscripts must be typed in double spacing (including figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. *Tables, Foot-Notes, Figure Legends, etc.* Tables should be typed on separate sheets and placed after the Literature Cited. Because of the high cost of setting such material in type authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. *A condensed title* or running head of no more than 35 letters and spaces should be included.

4. *Literature Cited.* The list of references should be headed LITERATURE CITED, should conform in punctuation and arrangement to the style of recent issues of THE BIOLOGICAL BULLETIN, and must be typed *double-spaced* on separate pages. Note that citations should include complete titles and inclusive pagination. Journal abbreviations should normally follow those of the U. S. A. Standards Institute (USASI), as adopted by BIOLOGICAL ABSTRACTS and CHEMICAL ABSTRACTS, with the minor differences set out below. The most generally useful list

Continued on Cover Three

THE BIOLOGICAL BULLETIN

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HISTOLOGICAL STUDIES ON THE PYLORIC STOMACH AND ITS APPENDAGES IN *OREASTER RETICULATUS* (L.) (ASTEROIDEA)

Reference: *Biol. Bull.*, **156**: 1-19. (February, 1979)

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The gross anatomy of the digestive system in the large tropical-Atlantic sea-star, *Oreaster reticulatus*, was recently described (Anderson, 1978), and attention was directed to several distinctive features of the system presumably developed in adaptation to various modes of feeding and the handling of diverse types of food. These features include, in addition to the large, broadly eversible cardiac stomach, strikingly complex Tiedemann's pouches and radial reservoirs associated with the paired pyloric caeca; unusually elaborate systems of folds in the roof of the pyloric stomach, in patterns converging on the opening into the intestine; and a set of conspicuously well-developed intestinal caeca. The parts of this system lying above the cardiac stomach constitute a good example of what Jangoux and van Impe (1971), describing conditions in *Asterina*, *Henricia*, and *Echinaster*, refer to as the "complexe stomacal superieur." It is the purpose of the present paper to describe histological details of various components of this complex in *Oreaster*, with particular attention to the specialized types of cells found in the epithelium lining the system and their specific localization in the various organs. Histological aspects will be correlated with anatomical characteristics already described at the gross level. The intestine, intestinal caeca, and rectum will not be considered here, nor will the cardiac stomach; the size and complexity of this organ, with its elaborate retractor mechanism, make it impractical to include this part of the system in the present account.

MATERIALS AND METHODS

Specimens were maintained, prepared, and dissected as previously described (Anderson, 1978). Portions of digestive organs were removed and fixed for two to four days in Bouin's solution. They were then given a prolonged soaking in repeated changes of 80% ethanol to remove picric acid, and dehydrated, cleared, and embedded in paraffin using standard histological methods. Tissue

blocks were subsequently sectioned serially at 7 to 10 μm . For general histological observations, sections were stained with Harris's hematoxylin and light green, or with Mallory's phosphotungstic acid hematoxylin (PTAH); the PTAH stain is excellent also for demonstrating secretory granules and for distinguishing between muscle and connective-tissue fibers. Glycogen and mucopolysaccharides were visualized by the periodic-acid-Schiff routine (PAS), with suitable diastase controls. The distribution, localization, and some characteristics of mucous secretions were further studied using metachromatic staining with dilute aqueous toluidine blue, as well as Mowry's alcian blue technique. Detailed procedures for all these staining routines are given in Lillie (1965). In addition to these, the aluminum-methylene-blue technique introduced by Heath (1962) was used to demonstrate sulfated mucopolysaccharides.

After appropriate preliminary treatment, some Bouin-fixed tissues were embedded in glycol methacrylate and the plastic polymerized, according to the directions of Feder and O'Brien (1968). Sections were cut at approximately 1 μm and stained with PAS, aqueous toluidine blue, methylene blue and eosin, or acid fuchsin and Harris's hematoxylin. Fine details of cellular structure and contents are particularly well displayed in such thin sections.

OBSERVATIONS AND RESULTS

The wall of the gut in *Orcaster* consists of the standard set of tissue-layers, described originally by Hamann (1885) for *Asterias rubens* and confirmed repeatedly, by subsequent investigators, in a number of other species. There is a visceral peritoneum, underlain by two layers of slender fibers representing, according to Hamann, longitudinal and circular muscles. Then follows a layer of connective tissue, the inner surface of which serves as a basement membrane supporting the epithelium lining the cavity of the gut. A layer of nerve fibers occupies arcades among the basal processes of the cells of the lining epithelium. Each of the layers making up the wall of the gut shows considerable variation in thickness, degree of development, and other characteristics, which can frequently be seen as related to local functions. It will be instructive to examine the general features of the principal layers before proceeding to a description of the histological characteristics of the digestive organs which they compose.

The visceral peritoneum

This is a simple, cuboidal, flagellated epithelium; it varies from about 7 to 35 μm in height, being tallest in folds and thinnest where it is stretched over ridges. Most of the cells of this layer contain no obvious cytoplasmic inclusions, but individual cells or groups of cells are frequently encountered in which the cytoplasm is packed with coarse, spherical granules measuring about 2.5 μm in diameter. These granule cells are conspicuous in sections because they are always somewhat taller than neighboring cells, and because their granular contents stain distinctively. They are intensely basophilic (orthochromatic) with toluidine blue or methylene blue at pH 6, give a negative or very faintly positive reaction with the Heath aluminum-methylene-blue routine, and stain deep purple

with PAS even after diastatic digestion. With PTAH the granules usually stain dark blue, but occasionally some or all of the contents of a single cell stain pink instead. Granule cells are often seen in sections apparently releasing either clumps of granules or a single, large, clear droplet into the coelom.

The connective-tissue layer

The characteristics of this layer agree in general with those described by Bargmann and Behrens (1968) for the corresponding tissue in the gut of *Asterias rubens*. It consists principally of a felty layer, varying in thickness, with inner and outer surfaces condensed to form basement membranes for the adjacent epithelia. Nuclei of connective-tissue cells are scattered in the felty layer. In *Oreaster* the fibers of this layer stain pink with PTAH; with the PAS routine, following diastatic digestion, the felty portion of the layer commonly shows a slight positive reaction, while the condensed surface layers are consistently strongly positive. The entire layer stains well with alcian blue, is faintly positive with the Heath stain, and shows a varying degree of metachromasia, always red-purple, with toluidine blue. These reactions are in general what could be expected of a connective tissue containing collagen (Mowry, 1963).

In the connective-tissue layer there are numerous lacunae which, followed through serial sections, prove to constitute an extensive network of branching channels confined to this layer. Although they are always empty of cells or coagulated fluid, it seems reasonable to interpret them as hemal vessels. Hamann (1885, p. 70) describes just such a hemal plexus in the connective-tissue layer of the gut wall in *Asterias rubens*.

The lining epithelium

This is the thickest layer in the wall of the gut and shows the greatest histological diversity. As described repeatedly by various authors for a number of species of asteroids, it is a tall, columnar, pseudostratified epithelium in which the cells are very closely crowded together. Variations in the thickness of the wall of the gut in various regions principally reflect differences in the height of this lining layer, which in *Oreaster* constitutes, in general, from 50 to 85% of the entire wall. The characteristics of the several types of cells present in this epithelium may be summarized as follows:

Typical cells. These constitute the basic type and are the most numerous and least specialized cells in the epithelium. Each cell bears a single flagellum and, in most regions, is provided with a well-developed brush border. The nuclei are ovoid to elongate, depending in part on the degree of crowding, and they lie in a band occupying about the middle third or more of the epithelium. Below this nuclear band the basal portions of the typical cells become very slender and gather into groups which proceed to attachments on the basement membrane. The arcades between these basal prolongations, taller in some parts of the gut than in others, are occupied by fibers of the nerve layer, or by other kinds of cells in the epithelium. The distal ends of the typical cells contain accumulations of fine granular material which shows a diastase-resistant coloration with PAS.

stains brightly with alcian blue and with the Heath technique, and is strongly metachromatic with toluidine blue. As will be seen, these properties resemble those of substances elaborated by some of the specialized secretory cells of the epithelium, although the typical cells share none of the other characteristics of such cells. With the PAS technique, the general cytoplasm of the typical cells gives a slight indication of glycogen storage, varying in different regions of the gut, but most of the PAS reactivity is not removed by diastase.

Zymogen cells. In the gut of *Oreaster*, as in many other asteroids, these cells are characterized by accumulations of uniformly-sized granules, frequently extending in long files from broader clumps at the base of the cell all the way to its distal end at the surface of the epithelium. As in other species, the granules are often, but not invariably, associated with a clear vacuole. The zymogenic nature of the secretory granules in such cells, long inferred on circumstantial grounds, has recently been definitively established by Hori, Tanahashi, and Matsuoka (1977), working with the pyloric caeca of *Asterias amurensis*. In *Oreaster*, zymogen cells are sparse or altogether lacking in some portions of the gut; where they do occur, their abundance varies considerably.

Mucous gland cells. These form a conspicuous and abundant component of the lining of the gut in *Oreaster*, as in other sea-stars. They may be recognized by the characteristic appearance and staining behavior of their secretion product; in Bouin-fixed material this is a frothy, vacuolate accumulation of varying shapes and dimensions. Scattered among typical cells, broad, spindle-shaped masses of secretion, 35 μm tall and 10 μm in greatest breadth, lie high in the epithelium, with a narrow neck extending to the surface. In other regions of the gut, where cells of this type are more numerous, the secretion masses are narrower and more cylindrical and traverse the entire thickness of the epithelium, which may be as great as 380 μm . In all cases, the secretion product of mucous gland cells in *Oreaster* gives a strongly positive reaction with the PAS technique, which is diastase resistant. The secretion product also shows brilliant metachromatic staining with toluidine blue, and stains brightly with both alcian blue and aluminum-methylene-blue. In the interpretation of several authorities, this pattern of staining reactions indicates that the substance is a sulfated acid mucopolysaccharide. Mowry (1963), for example, states that alcian blue colors PAS-positive acid mucopolysaccharides that also stain metachromatically with toluidine blue; Heath (1962) considers his aluminum-methylene-blue method specific for sulfated mucopolysaccharides; and Barka and Anderson (1963, p. 86), state that the strongest alcohol-resistant metachromasia is given by sulfated acid mucopolysaccharides and is almost diagnostic for these compounds. It is worth noting that in many regions of the gut the brush border appears to be coated with material showing the same staining properties, and similar material, presumably released from the mucous gland cells, has evidently been precipitated among the flagella of the typical cells.

Globule cells. These constitute another type of secretory cell in the lining of the gut in *Oreaster*. I cannot establish their correspondence to any previously described type of cell in the asteroid digestive system, and I give them this name because of the characteristic appearance of their secretion product. Each cell is closely packed with coarse globules which are much larger than the granules found in zymogen cells. Unlike zymogen granules, they stain pink rather than

blue with PTAH; and unlike the contents of the mucous gland cells, these globules are unstained by either alcian blue or the Heath process. Further, they are orthochromatically basophilic with toluidine blue, and where globule cells lie among mucous gland cells in the epithelium, as they commonly do, this stain shows the bright blue globules in brilliant contrast to the purple mucus. Their reaction with the PAS technique is peculiar: the globules themselves are PAS-negative and are colored by the counterstain; associated with each globule, however, are small, bean-shaped, PAS-positive bodies which are not affected by diastatic digestion. With this stain the cells thus show a curious speckled appearance, but the small PAS-positive bodies are unstained by alcian blue, toluidine blue, or the Heath technique. The distribution of globule cells in association with mucous gland cells suggests that they may represent precursors of these cells, or a different but related type of cell; on the other hand, their secretions may be completely unrelated to mucosubstances.

Basal cells. These complete the list of cell types found in the lining of the gut. Although they are variable in size, form, and contents, it is assumed here that they represent a single class of cell. Basal cells occur everywhere in the gut, in widely varying abundance. Typically, they lie at the base of the epithelium, among the proximal ends of the typical cells or other components; but in some regions they become so numerous that they crowd almost up to the surface, displacing the nuclear band upward or even penetrating beyond it. Basal cells appear in two basic forms, with some intergradation: one form contains a large, clear, apparently empty vacuole, while in the other the vacuole is partially or completely filled with granular or irregularly flocculent material. In either form the cell has a small, dense nucleus about $2.5\text{ }\mu\text{m}$ in diameter. The granular material accumulated by the cell stains blue with PTAH and pink with acid fuchsin, is always PAS-positive, and does not stain with alcian blue or the Heath technique: at pH 6 it stains orthochromatically with methylene blue or toluidine blue.

In every asteroid studied, the digestive system shows as one of its most conspicuous characteristics a consistent pattern of distribution of specialized types of cells, and combinations of types, so that each region can be recognized by its histological features. The gut of *Oreaster* furnishes excellent examples of this regional specialization, which will now be examined. To avoid the necessity of tiresome repetition, it will be helpful to refer to the recent description of the gross anatomy of this system (Anderson, 1978) for orientation.

Tiedemann's ducts are cylindrical tubes proceeding distally in pairs from the lowest part of the pyloric stomach. Until it opens out to form the oral gutter of the corresponding Tiedemann's pouch, each duct is separated by a permanent seam-like partition from the overlying part of the pyloric stomach known as a radial reservoir, to which it is attached. The lining of the duct shows a number of histological peculiarities. This is one of the regions, referred to earlier, in which the typical cells are extremely crowded and distorted by masses of basal cells. In the floor of the duct these cells, filled with granules, occupy the lower two-thirds of the epithelium, which also contains numerous mucous gland cells (Fig. 1). In the side walls one sees a series of four or more rounded ridges, running longitudinally, in which the epithelium rises to a thickness of about $250\text{ }\mu\text{m}$; these are separated by valleys where it is $100\text{ }\mu\text{m}$ tall, or less. As shown

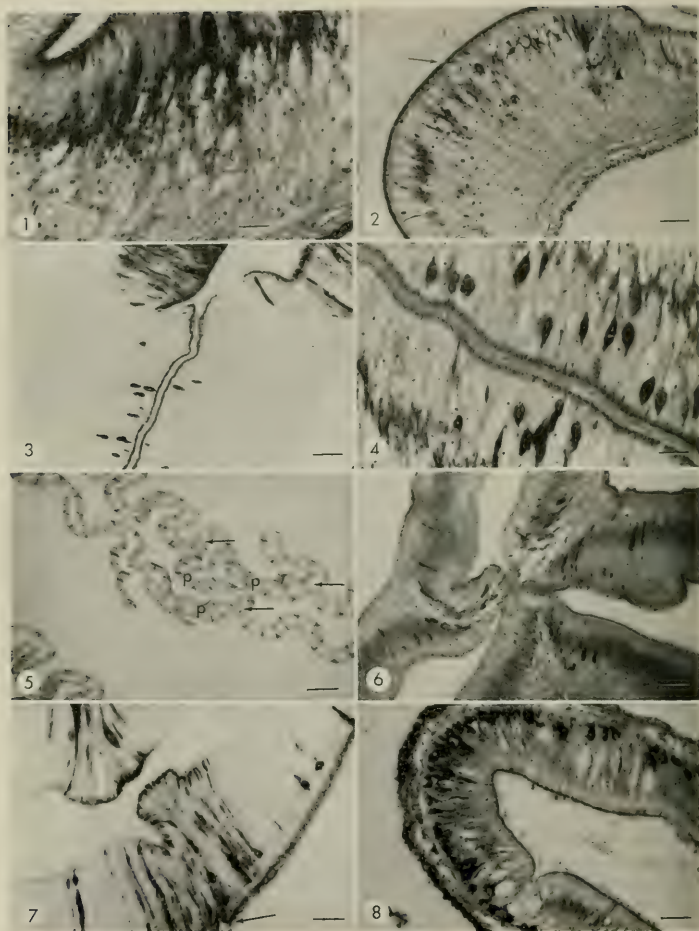


FIGURE 1. Cross-section of the floor of Tiedemann's duct; lumen of duct at upper left, visceral peritoneum at lower right (alcian blue, Harris's hematoxylin). The distal granules in the typical cells near the lumen, the contents of the large, spindle-shaped mucous gland cells, and the subepithelial connective-tissue layer stain with alcian blue. Granules in the masses of basal cells, together with their small, dense nuclei, stain with hematoxylin. Scale bar = 20 μ m.

in Figure 2, the integrity of the epithelium in the ridges is markedly disorganized by the great abundance of granule-filled basal cells, which in these areas extend all the way to the surface and in many cases appear to be discharging their contents into the lumen.

The epithelium responsible for maintaining the permanent separation between the lumen of the duct and that of the overlying radial reservoir is composed of the same elements found in the walls of the duct. There is no zone of transition; the surfaces of the two facing epithelia simply approach each other and adhere to form the partition. Proximally, the zone of adhesion is a millimeter or so broad, and it becomes progressively narrower distally, finally disappearing as the duct opens to form the oral gutter. Figures 3 and 4 show some of the features of the seam. Mucous gland cells are abundant, lying high in the epithelium above the nuclear band of the typical cells. A large number of basal cells is also present, with contents varying from empty vacuoles to crowded granules; these cells also extend in many cases to the surface of the epithelium. The distal

FIGURE 2. Cross-section of a longitudinal ridge in Tiedemann's duct (alcian blue, Harris). Note again the alcian blue-positive deposits below the brush border in typical cells. The basal cells, variously granular and vacuolate, lie in vertical columns reaching the lumen, where (as indicated) discharge into the lumen appears to occur. A few granule-filled cells can be seen in the peritoneum. Scale bar = 40 μ m.

FIGURE 3. Cross-section showing the transition zone between the lining of a radial reservoir (above) and the facing epithelia that have joined to form the horizontal partition at the top of Tiedemann's duct (Heath's aluminum methylene-blue). Mucous gland cells are selectively stained, both in the seam area and in the radial reservoir, as are the distal granules. No Heath-positive material is involved in the adhesion between the epithelial surfaces (compare Fig. 4). Scale bar = 40 μ m.

FIGURE 4. Detail of the zone of adhesion in the horizontal partition (alcian blue, Harris). Tissue elements are stained as in Figure 1; the interdigitating brush-border filaments are alcian blue negative, staining with hematoxylin. Scale bar = 20 μ m.

FIGURE 5. Frontal section showing the relationship between the principal and accessory Tiedemann's pouches (dilute aqueous toluidine blue). The principal pouch (*p*) zigzags, giving off accessory pouches alternately to right and left. The serially repeated dark patches represent vertical ridges of specialized tissue, occurring in matched sets in the facing epithelia. Junctions between principal and accessory pouches (indicated) show sets of three rather than two ridges (compare Figure 6). Scale bar = 450 μ m.

FIGURE 6. Detail of a three-ridge partition marking the origin of an accessory Tiedemann's pouch (PAS, Harris). The lines of junction between the three ridges show a thin layer of PAS-positive material, staining like the conspicuous mucous gland cells in the epithelium. The clear areas in the center of each ridge consist principally of vacuolate basal cells. Scale bar = 40 μ m.

FIGURE 7. Detail of the epithelium in a pair of facing ridges in Tiedemann's pouch (dilute aqueous toluidine blue). All darkly staining elements are strongly metachromatic, including distal granules at the summit of the ridge, elongate mucous gland cells, and the connective-tissue layer (containing a hemal channel indicated by the arrow). Paler secretory elements flanking the ridge are orthochromatic globule cells; a large basal cell in the center of the lower ridge is filled with basophilic granules, while in the upper ridge a vacuolate basal cell appears as an empty space. Scale bar = 20 μ m.

FIGURE 8. Section of the oral gutter of an accessory Tiedemann's pouch (PTAH). Note the conspicuous distal granular deposits in the typical cells, the masses of granular basal cells, and the accumulation of granule-filled cells in the peritoneum outside the gutter. Clear areas in the lining epithelium are mucous gland cells; those in the connective-tissue layer are hemal channels. A few files of zymogen granules are seen in the lining epithelium. Scale bar = 40 μ m.

ends of all the typical cells are filled with mucopolysaccharide granules or droplets. Flagella are lacking, but the brush borders of both epithelia, well developed, interdigitate with each other and evidently play an important part in holding the two surfaces in contact. Interestingly, the line of junction between the brush borders does not exhibit the staining characteristics of mucus or other mucosubstances, as might have been expected, given the abundance of mucous gland cells in the epithelia; the brush-border elements adhering to each other at this line are instead faintly basophilic.

In *Oreaster*, Tiedemann's pouches are noteworthy for their complexity, each possessing a large series of accessory pouches which branch off laterally in alternating fashion and show, like the principal pouch, parallel vertical striations in the walls, as viewed from outside. Figure 5 gives some idea of the zig-zag arrangement of these thin-walled, narrow pouches and reveals also the nature of the striations. Each marks a distinct band of thickened epithelium, histologically very different from the lower intervening areas; the bands in opposite walls face each other in sets or pairs. Each set is evidently capable, in life, of forming an adhesion, or vertical partition, separating narrow parallel channels. There is no indication that these adhesions are permanent; they very frequently pull apart during histological processing without tearing the cells involved. The channels formed by the ridges, lined by typical cells and closed off from their neighbors on both sides, presumably provide enhanced efficiency in the movement of fluids upward from the principal and accessory oral gutters to the overlying glandular pockets of the pyloric caecum. It may be noted that, for an effective partition at the line of origin of an accessory pouch, a set of three, rather than two, ridges is required (Figs. 5, 6).

Figure 7 illustrates the marked contrast in histological composition between a band or striation and the epithelium on either side. Most notable are the absence of flagella and brush borders from the cells at the summit of the ridge, and the presence in the upper ends of these cells of large numbers of granular deposits. These are of two sorts: smaller granules, in the extreme distal ends of the cells, that have the staining properties of sulfated mucopolysaccharides; and coarser granules just below these, in smaller numbers, that are yellowish brown in color and do not stain at all. The distal granules resemble those usually observed in typical cells elsewhere in the gut, although it is to be noted that they are not present in the typical cells lining the channels. Their abundance in the tips of the cells at the ridge-summits suggests that they have to do with adhesion across the lumen. In preparations where the cells remain in contact (Fig. 6), the line of junction stains as though a thin layer of mucus were present. The central core of the ridge is occupied by numbers of basal cells, either vacuolate or granular; the granules, if present, do not stain like those in the distal parts of typical cells. Flanking the central region are numerous tall, slender, vacuolate mucous gland cells, along with some globule cells. Mucous gland cells are found in modest numbers among the typical cells lining the channels, but they are never so numerous in these areas as on the flanks of the elevated ridges. Figure 7 shows one additional feature of the striations: each is usually accompanied by at least one hemal channel, and frequently two or three, running in the subepithelial connective-tissue layer at its base.

The accessory Tiedemann's pouch tapers upward and disappears, but its oral gutter continues outward below the midline of the series of lateral diverticula which evaginate to either side, collectively making up one of the glandular pockets of the pyloric caecum. Some details of the histology of such a gutter are seen in Figure 8. There are strong resemblances to the epithelium in the floor of Tiedemann's duct (Fig. 1), and to the oral gutter of the principal Tiedemann's pouch: many basal cells, packed with granular material, are crowded among the bases of the typical cells, and mucous gland cells are numerous. Here, however, zymogen cells are encountered for the first time. The nerve layer is comparatively thick beneath the epithelium at the bottom of the gutter, and there is a noticeable accumulation of granule-filled peritoneal cells in this area as compared with adjacent side walls. The epithelium lining the side walls does contain an abundance of mucous gland cells, as seen in Figure 9.

The histological features of the glandular pockets of the pyloric caecum have been described for a number of species by various authors (Anderson, 1953, 1960, 1966; Mauzey, 1966; Chia, 1969; Nimitz, 1971; Jangoux and Perpeet, 1972), and this portion of the digestive system in *Oreaster* does not differ sufficiently from corresponding parts in other forms to warrant detailed description here. Figure 10 is included to illustrate the extensive folding of the wall involved in the formation of the lateral diverticula; Figure 11 shows something of the number of zymogen cells and mucous gland cells, as well as hemal channels, in the wall of a diverticulum. In these organs the typical cells have become modified to serve as absorptive and storage cells, and scattered coarse granules or droplets are present. Such cells show a positive reaction with PAS, but much of the material responsible for this is diastase resistant; perhaps most of the stored carbohydrate is in a form other than glycogen, a situation which has been noted in other asteroids as well. The storage of lipids in *Oreaster* was not investigated.

It will be recalled that organs referred to as radial reservoirs, which are radiating branches alternating with deep indentations of the walls of the pyloric stomach, lie at the proximal ends of the pyloric caecum-Tiedemann's pouch complexes. Each is bounded below by the seam forming the roof of the corresponding Tiedemann's duct and is continuous above with the root of the median duct of the pyloric caecum. The epithelium in the radial reservoirs is tall, ranging up to 250 μm , and it shows a number of characteristic and unique features. Although brush border and flagella appear to be present, it is difficult to distinguish typical cells: the epithelium contains such large numbers of mucous gland cells that other normal components are crowded almost beyond recognition. As shown in Figure 12, some granule-filled basal cells are to be found at the base of the layer, and files of granules extend upward from this level; whether these represent basal-cell granules or zymogen granules cannot be determined, although, by analogy with the corresponding tissue in *Hemicidia*, zymogen cells should be abundant here (Anderson, 1960). Nuclei are scattered in a wide band and are compressed to slender, spindly forms (Fig. 13). The preponderance of mucous gland cells with their contained secretion product causes the entire epithelium to give a very strongly positive staining reaction with

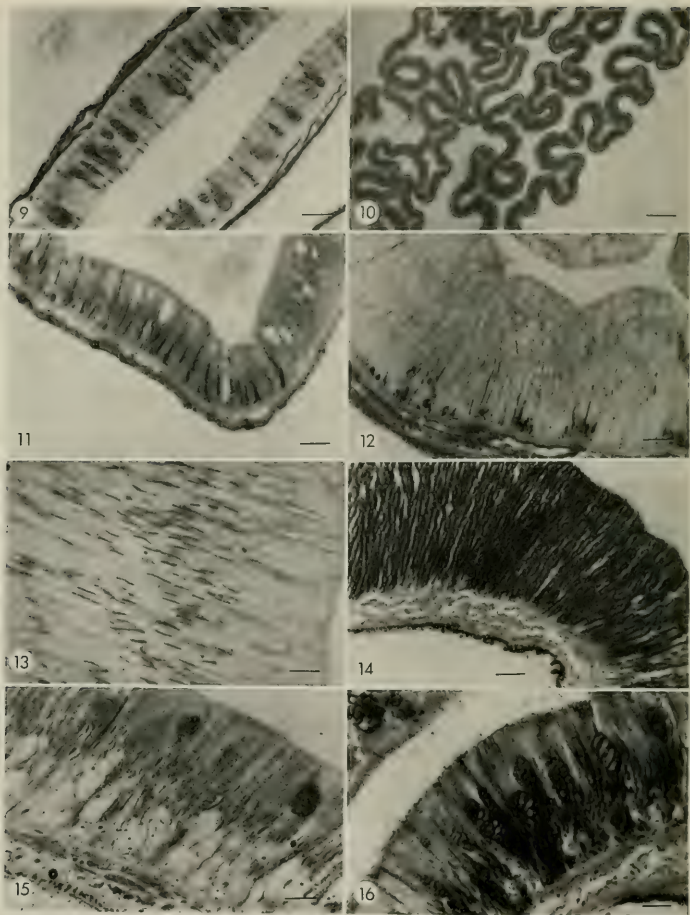


FIGURE 9. Frontal section of the walls of an accessory Tiedemann's pouch, above the level where the parallel vertical ridges disappear (dilute aqueous toluidine blue). Note the vivid metachromatic staining of the frothy mucous gland cells, and of the connective-tissue layer (containing numerous hemal channels). Orthochromatic globule cells, seen particularly in the lower epithelium, show up less clearly. Scale bar = 40 μ m.

FIGURE 10. Frontal section of two adjacent glandular pockets of the pyloric caecum, showing the extensive folding of the walls that forms the numerous lateral diverticula (Harris, fast green). Scale bar = 250 μ m.

all procedures designed to demonstrate mucus. The overall appearance of the tissue after staining with alcian blue is shown in Figure 14, where it is clear that the cells remaining unstained are vastly outnumbered by those staining bright blue. Radial reservoirs at least superficially similar to those of *Oreaster* have been described in *Henricia* (Anderson, 1960) and in *Linckia* (Anderson, 1966).

At the bottom of the radial reservoir there is a sharp and sudden transition between its lining and the epithelium forming the seam; an indication of this can be seen in Figure 3. At the upper boundary of the reservoir there is a much more gradual shift to a different kind of epithelium, which is characteristic of the median duct of the pyloric caecum. The overall thickness of the gut wall is increased in this region, partly by a thickening of the connective-tissue layer. This receives contributions from the horizontal connective-tissue sheet attached to the outside of the gut wall at this level and forming part of the complex harness suspending the digestive organs from the aboral body wall. The lining here is most diverse, containing representatives of every type of cell that occurs anywhere in the epithelium of the gut. Figures 15 and 16 show its general nature. Mucous gland cells are abundant, but much less so here than in the radial reservoirs. Interspersed among them are large numbers of globule cells, as well as many zymogen cells. The lower half of the epithelium is filled with basal cells, varyingly vacuolate or granule-packed. Typical cells provide support for the secretory types and furnish brush border and flagella, although these elements are not strongly developed in this region.

FIGURE 11. Section of the outer end of a glandular pocket of the pyloric caecum (PTAH). Dark-staining zymogen cells and unstained mucous gland cells are abundant in the lining epithelium. The connective-tissue layer contains numerous hemal channels, and several granule-filled cells appear in the peritoneum below. Scale bar = 40 μ m.

FIGURE 12. Section of the wall of a radial reservoir (PTAH). The epithelium appears relatively clear, because it consists mainly of mucous gland cells, which are stained pale pink with this technique. Granule-filled basal cells lie in masses at the base of the epithelium, and linear arrays of granules at various levels are interpreted as zymogen cells. Scale bar = 40 μ m.

FIGURE 13. Detail of the lining epithelium of a radial reservoir (thin section, Harris's hematoxylin and acid fuchsin). The free surface of the epithelium is at the right edge of the picture. The nuclei of both mucous gland cells and typical cells are crowded and squeezed by the accumulated masses of mucous secretion. Scale bar = 20 μ m.

FIGURE 14. Section of the wall of a radial reservoir (alcian blue, Harris). The preponderance of brightly staining mucous gland cells is noteworthy; intervening clear areas presumably represent typical cells and zymogen cells remaining unstained in this technique. Scale bar = 40 μ m.

FIGURE 15. Section of the side wall of the median duct of a pyloric caecum, as it enters the pyloric stomach above the corresponding radial reservoir (thin section, PAS, Harris's hematoxylin). Note the diversity of tissue elements in the lining epithelium. The large, dark masses are mucous gland cells; vacuolate, partly granule-filled basal cells occupy the entire lower level of the tissue. The elongate, speckled elements running up to the surface are globule cells, in which the globules are lightly stained by the hematoxylin, while accompanying small, dense bodies in the cell are PAS-positive. Typical cells send groups of slender basal prolongations down to the basement membrane, through fibers of the nerve layer. Scale bar = 20 μ m.

FIGURE 16. Section of an area similar to that shown in Figure 15 (alcian blue, Harris). In this region the frothy mucous gland cells are numerous; in the accompanying globule cells, it should be noted that neither the globules nor the small, dense bodies stain with alcian blue. Scale bar = 20 μ m.

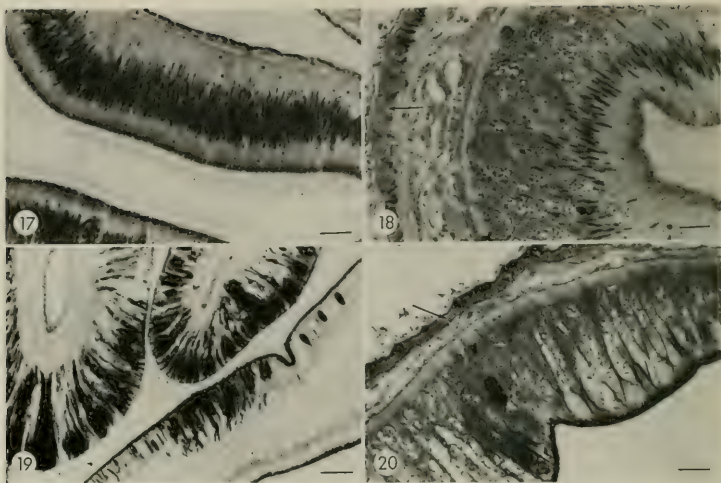


FIGURE 17. Section of the wall of a side branch of a fold-pattern in the roof of the pyloric stomach. The top of the fold or inverted gutter lies to the right (alcian blue, Harris). Note the relative simplicity of the epithelium, here consisting mainly of typical cells, with brightly staining, alcian blue-positive distal granules, and conspicuously long flagella. In many similar areas basal cells are much more numerous than in this section. Scale bar = 20 μ m.

FIGURE 18. Thin section of the top of a fold in one of the sets of patterns in the roof of the pyloric stomach (Harris's hematoxylin, acid fuchsin). The abundance of granular basal cells is striking; beyond the thick connective-tissue layer, with its hemal channels, is a characteristic accumulation of granule-filled peritoneal cells (arrow). Scale bar = 20 μ m.

FIGURE 19. Section showing inward extensions of glandular epithelium on downhanging folds between the inverted gutters in the roof of the pyloric stomach (dilute aqueous toluidine blue). Strong metachromatic staining is seen in the mucous gland cells, and in the distal granules of typical cells (compare Figure 17). Orthochromatic globule cells lie among the mucous gland cells. Scale bar = 40 μ m.

FIGURE 20. Section near the midline of the main channel of a fold pattern; this channel leads directly toward the opening into the intestine (thin section, methylene blue and eosin). Columnar arrays of granule-filled basal cells occupy almost the entire thickness of the lining epithelium, as in the floor of Tiedemann's duct (compare Fig. 1). The nerve layer and connective-tissue layer are thick, and the longitudinal muscle layer (arrow) is much better developed here than elsewhere in the gut. Granule-filled peritoneal cells become much more numerous just at the midline of this main channel. Scale bar = 40 μ m.

A type of epithelium very similar to that just described covers the roof and side walls of the median duct of the pyloric caecum, or, as it may be called at this level, the pyloric duct. Conspicuous longitudinal ridges are present, alternating with areas where the lining is much thinner. Mucous gland cells and globule cells are the predominant secretory type.

As noted earlier, the roof of the pyloric stomach is provided with ten sets of branching fold-patterns, resembling inverted gutters; one of these sets lies between the proximal end of each pyloric duct and the opening of the intestine. In each set the folds, which distally are spread rather broadly, converge toward the center, ultimately forming a single major channel, open below, running toward the intestine. In the lining of the stomach there is a gradual transition from the diverse epithelium of the pyloric duct, just described, to a much simpler tissue in the roof folds. In the lateral branches of the fold-patterns, the lining consists of a tall epithelium made up principally of typical cells, with well-developed brush border and unusually long flagella (Fig. 17). In some areas there are large accumulations of vacuolate basal cells. At the tops of the inverted gutters in this region, as seen in Figure 18, the connective-tissue layer and the nerve layer are much thickened; here the basal cells in the epithelium tend to be granule-filled rather than vacuolate, although this is variable. Mucous gland cells are scattered in the epithelium of the roof folds, but such cells, with their accompanying globule cells, are chiefly concentrated on the downhanging folds between inverted gutters (Fig. 19). Here, in effect, the lining represents inward extensions of the tissue covering the roof of the pyloric duct, except that zymogen cells are sparse in the folds.

Figure 20 shows a section near the midline of the roof of one of the major channels of a fold pattern, leading toward the intestine. Notable here is the great concentration of basal cells in the lining, not confined to its lower portion but extending upward to just below the free surface. In some ways the epithelium here resembles that observed on the longitudinal ridges in Tiedemann's duct (compare Fig. 2). In both locations, the basal cells seem to move up in columns between partitions formed by groups of typical cells. In the roof, the typical cells, disarranged as they are, contain the usual distal deposits of basophilic, metachromatic granules beneath the brush border. Flagella are present, but in the main channel they are never so long and numerous as in the lateral branches of the fold patterns; this may simply reflect the fact that the proportion of flagellated typical cells in the roof of the major channel is reduced by the great accumulation of basal cells. The nerve layer and connective-tissue layer are both relatively thick, and the layer of longitudinal muscle (but not the circular ones) just under the peritoneum is markedly hypertrophied. Hemal channels are well developed in the connective-tissue layer. It should be noted, however, that the superficial, parallel, longitudinal structures, tentatively identified previously as probable hemal channels (Anderson, 1978, p. 5 and Figs. 7, 8), are not, after all, parts of the hemal system. Instead, they are revealed in sections as consisting only of thick, upthrust folds of connective tissue, covered externally by the muscle layers and granule-filled peritoneal cells, which are unusually abundant throughout their length. The functional significance of these structures, present in association with the major channel in each of the roof-pattern sets, is unknown.

DISCUSSION

The detailed picture of the histology of digestive organs in *Oreaster* that emerges from the kind of study presented here can be usefully compared with

what has been found in other sea-stars for which similar results are available. To be most meaningful, comparisons should probably not involve the various asteriids that have been studied, because in these the general morphological adaptations of the digestive system are very different from those of *Oreaster*. We should concentrate instead on whatever information is at hand concerning species that have well-developed Tiedemann's pouches, with or without radial reservoirs, in association with the pyloric caeca. It is unfortunate that histological details have evidently never been published for forms such as *Porania pulchillus*, or any of the close relatives of *Oreaster* such as *Culeita*, in which the digestive organs approach those of *Oreaster* in complexity. Information is available, however, for *Patiria miniata*, which has large Tiedemann's pouches, and for *Henricia leviusecula*, which has both pouches and radial reservoirs.

In earlier discussions (Anderson, 1960, 1966), I have pointed out that, in the asteroid digestive system, organs resembling each other superficially, and probably serving the same or very similar functions, are not necessarily identical in histological composition. For example, Tiedemann's pouches in *Patiria* are broad and baglike; no horizontal partitions are present to separate outflowing and inflowing currents, and the parallel vertical striations in the side walls consist of linear accumulations of mucous gland cells evidently capable of forming loose, temporary adhesions across the lumen. In contrast, *Henricia* has deep, narrow pouches, with a permanent seam dividing Tiedemann's duct from the lumen of the overlying radial reservoir, and with parallel channels in Tiedemann's pouch maintained by bands of permanently fused, specialized cells in the opposing epithelia. From the description presented earlier, it can be seen that *Oreaster* exhibits yet another pattern: the radial reservoir is effectively sealed off from Tiedemann's duct by a permanent seam, but this structure is much different in detail from the corresponding feature in *Henricia*. Further, the parallel striations in the walls of the principal and accessory Tiedemann's pouches differ from both the temporary mucous-gland partition in *Patiria* and the permanent seams in *Henricia*. It is evident, and interesting, that similar functional problems have been solved in different but convergent ways by these three diverse genera. Such seams or partitions have not, to my knowledge, been described in other echinoderms, although it is highly probable that they occur in all species that are closely related to the ones we have been discussing. The exact nature of the permanent fusion between facing epithelia in *Henricia* has not been closely examined; investigations into the fine structure of this unique cell-to-cell junction should yield interesting results.

Like the partitions in Tiedemann's pouches, the radial reservoirs in *Oreaster* are different in detail from those in *Henricia*, the only other form in which their histological features have been described, although the organs are generally similar in appearance. In *Henricia* (Anderson, 1960), the walls of the reservoir contain a very high concentration of zymogen cells, an extension of the similar condition in the roof and side walls of the median duct of the pyloric caecum. In *Oreaster*, in strong contrast, the predominant type of cell in the lining of the radial reservoir is the mucous gland cell, with zymogen cells and even typical cells present in much smaller numbers. In *Oreaster* the lining of the median duct

is not like that of the radial reservoir. Instead, as described earlier, it contains an abundance of globule cells, as well as mucous gland cells in reduced numbers; this type of epithelium extends into the roof of the pyloric stomach, between the branches of the inverted fold-patterns.

The importance of mucous secretions in the digestive physiology of *Oreaster* is suggested by the wide distribution of mucous gland cells in various parts of the digestive tract, as well as by their extreme local abundance, almost to the exclusion of other types, in the walls of the radial reservoirs. The general appearance and staining reactions of their secretion product indicate that the mucous gland cells in *Oreaster* correspond to cells described under the same name in the gut of *Henricia* (Anderson, 1960), and in the digestive systems of *Patiria miniata* and *Pisaster ochraceus* (Nimitz, 1971). They are evidently similar also to the "mucocytes" or "cellules glandulaires spumeuses" observed by Jangoux and Perpet (1972) in the pyloric caeca of *Marthasterias glacialis*, *Coscinasterias tenuispina*, and *Asterias rubens*, and in both cardiac and pyloric stomachs of the last species by Jangoux, Perpet, and Cornet (1972). It is interesting that, in all asteroids studied, the digestive epithelium is said to contain only a single type of mucous gland cell. This is in contrast to the situation in echinoids, in which all investigators agree there are several types, differing in the chemical properties (and therefore the staining behavior) of their secretion products, as well as in their patterns of distribution in the gut (see, for example, Holland and Nimitz, 1964; Buchanan, 1969; Tokin and Filimonova, 1977). One curious fact emerges from a comparison of the results of these investigators: apparently none of the mucosubstances produced by any of the secretory cells in the three regular echinoids they studied shows the same combination of staining reactions as that characteristic of the secretion of the mucous gland cells described in the asteroids listed above. Instead, they show practically every other possible combination of reactions with PAS, alcian blue, and toluidine blue or azure A (and in some cases the Heath stain), but not the positive staining with PAS, alcian blue, and Heath, combined with toluidine blue metachromasia, that is seen in asteroid mucous gland cells. Whether this has any real significance, or is an artifact resulting from differences in technique or interpretation, remains to be determined.

It was suggested earlier that the globule cells in *Oreaster* might be somehow related to mucous gland cells. There is nothing really diagnostic in the staining reactions of their contents, at least at the level at which I have studied them, that can be used to establish such a relationship with any assurance. The globules, it will be recalled, differ from the frothy contents of the mucous gland cells by being PAS-negative and non-metachromatic, and by failing to stain with either alcian blue or aluminum-methylene blue. There are small PAS-positive bodies in the cells, however, which are not colored by the other stains. In these general properties the globules show some resemblance to the "type 2 mucous gland cells" in the radial buccal epithelium of *Strongylocentrotus purpuratus*, as described by Holland and Nimitz (1964), in their thorough study of gut mucopolysaccharides in this species. It may be, then, that *Oreaster*, unlike other sea-stars studied, does possess a second type of gland cell producing a mucus-related secretion; but this should by no means be taken as firmly established without better histochemical evidence bearing on the nature of the globules.

Unfortunately, the classification and identification of mucosubstances on the basis of histochemical staining reactions appears to be in a somewhat confused state. Contributing to this situation is the fact that the basic research has involved only vertebrate, chiefly mammalian material, and as recognized by Holland and Nimitz (1964), there is no reason to expect precise correspondence in properties of substances from these sources and those found in diverse invertebrate forms. Further, there are inconsistencies in the interpretations of different authorities analyzing vertebrate material. For example, Mowry (1963) cites results of an exhaustive series of experiments by Lillie as having demonstrated that virtually all epithelial mucins (one exception) are PAS-positive. Barka and Anderson (1963), in contrast, present a table (p. 68) indicating that acid mucopolysaccharides are PAS-negative. Using the criteria of Spicer (1963), Holland and Nimitz conclude that the product of their "type 2 mucous gland cell" in *Strongylocentrotus purpuratus* is a sulfated acid mucopolysaccharide. Its staining properties (alcian blue negative, orthochromatically basophilic, variably positive or negative with PAS) are obviously much different from those of the mucous gland secretion in *Oreaster*, which, using the criteria of Mowry (1963) and others, is interpreted in the present paper as a sulfated acid mucopolysaccharide.

The nature and significance of the granular inclusions in cells of the visceral peritoneum remain without elucidation. Peritoneal cells containing granules have been described previously in *Henricia leviuscula* (Anderson, 1962), in *Marthasterias glacialis* and *Coscinasterias tenuispina* (Jangoux and Perpeet, 1972), and in *Hippasteria phrygiana* and *Ctenodiscus crispatus* (Walker, 1976). It is apparent that in different species these cells, although similar in appearance, may not be identical: the granules contained in the "*cellules glandulaires granuleuses*" described by Jangoux and Perpeet are acidophilic, rather than basophilic as in other forms.

A final unresolved problem in considering the histology of the gut in *Oreaster reticulatus* involves the basal cells in the lining epithelium. I have assumed that they correspond to the cyst-like bodies first described in the cardiac stomach of *Patiria miniata* by Anderson (1959) and found also in the Tiedemann's pouches of the same species by Nimitz (1971). They evidently either do not occur, or have been overlooked, in other asteroids that have been studied. Their restriction to these two species (and possibly their close relatives, as yet unexamined) leads one to look for some peculiar feature common to both to which the presence of basal cells might be somehow related. It may be significant that both *Patiria* and *Oreaster*, as part of their normal feeding behavior, spend much of their time with the cardiac stomach broadly everted against the substrate (Anderson, 1959, 1978). Basal cells are particularly numerous in the cardiac stomach of *Patiria*, and they are also very abundant in the lining of the cardiac stomach of *Oreaster* (personal observations, unpublished). These facts may, of course, be entirely unrelated; further study should shed more light on the situation.

In *Oreaster* there are indications that in those parts of the gut where basal cells pile up in the lining until they reach the surface, they may be discharging their contents into the lumen. Such local accumulations are conspicuous, for example, on the ridges in Tiedemann's ducts, and particularly in the major

channels in the fold patterns in the roof of the pyloric stomach, leading toward the intestine. Although flagella are scanty in both places, strong currents are maintained by typical cells in adjacent areas where flagella are more numerous or better developed. Material conveyed to the lumen by basal cells would thus be rapidly distributed through the digestive system, or carried immediately to the intestine for possible elimination.

Detailed anatomical and histological studies on the digestive tract should continue and be extended to other genera of sea-stars, in diverse families, so that more significant and broadly comparative surveys may be made than are now possible with the limited information available.

Dissections, anatomical observations, and some histological procedures were carried out at the Mote Marine Laboratory in Sarasota, Florida. I am grateful for the many helpful kindnesses extended to me by the then Director of the Laboratory, Dr. Perry W. Gilbert (since retired), and by members of his staff. It is a pleasure to acknowledge also the skilled technical assistance of Mr. Anthony L. Guerriere of Cornell University.

SUMMARY

This paper presents a detailed histological study of those parts of the digestive system of *Oreaster reticulatus* lying between the upper limits of the cardiac stomach and the beginning of the intestine. In all parts, the wall of the gut consists of the same tissue layers found in other asteroids studied. Of these layers, the lining epithelium is histologically most diverse, containing the following types of cells: typical cells, most common and least specialized, with brush border and flagellum; zymogen cells, here as in other asteroids filled with secretion granules often associated with a vacuole; mucous gland cells, containing a frothy, vacuolate mass of secretion showing characteristic staining reactions; globule cells, in which the secretion consists of coarse, spherical globules staining in such a way as to suggest that they may represent a mucosubstance different from the product of the mucous gland cells; and basal cells, of unknown significance, often present in crowded masses in the epithelium, containing either a clear vacuole or an accumulation of granular material. All of these types of cells are present in the various digestive organs in characteristic patterns of distribution and combination, and these patterns have been described in detail for Tiedemann's duct and pouches, the glandular pockets and median duct of the pyloric caecum, the radial reservoirs, and the sets of fold-patterns in the roof of the pyloric stomach. It is noted that although some parts of the system, such as Tiedemann's pouches with their parallel wall striations, and the radial reservoirs, may resemble each other superficially in different species, close histological comparison shows important differences. Judging from staining behavior, mucous gland cells appear to be similar in all asteroids that have been studied, including *Oreaster*, but the properties of the secretion-product of these glands seem to differ from those of related substances produced by any of the several types of mucous gland cells found in the digestive systems of echinoids. The basal cells in *Oreaster* are believed to

correspond to cyst-like bodies, previously described, occurring in similar locations in the lining of the gut in *Patiria* but apparently not in other asteroids studied.

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REPRODUCTION AND LARVAL DEVELOPMENT OF *POLYDORA GIARDI* MESNIL (POLYCHAETA: SPIONIDAE)

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The polychaete family Spionidae is well represented in coastal marine environments and includes numerous species of the *Polydora*-complex. Larvae of this group are frequently encountered in neritic plankton and to date the larval development of over 25 species is known.

Important works on the larval development and reproductive biology of species of the *Polydora*-complex are by Wilson (1928), Hannerz (1956), Blake (1969), Woodwick (1960; 1977), Dean and Blake (1966), and Blake and Woodwick (1975). The majority of larvae described by these authors and others come from Europe, the east coast of North America and California.

Polydora giardi Mesnil is an inhabitant of coralline algae and abalone shells along most of the California coast (Hartman, 1969; Blake, 1975). Members of this species bore tunnels into these calcareous substrata and are often found in dense aggregations. The species was originally described from France (Mesnil, 1896) and subsequently reported from other European localities (Fauvel, 1927; Colman, 1940). The species was reported from California by Hartman (1941), from New Zealand by Ranier (1973) and Read (1975) and from Australia by Blake and Kudenov (1978).

There are no published accounts of larval development for *P. giardi*, although Mesnil (1896) determined his populations to be simultaneous hermaphrodites. The present paper describes aspects of the reproductive biology and larval development of *P. giardi* and presents descriptions and illustrations of developing larvae and recently metamorphosed juveniles.

MATERIALS AND METHODS

Egg capsules of *Polydora giardi* were collected from encrusting coralline algae, *Lithothamnium pacificum* Foslíe and occasionally from shells of the red abalone, *Haliotis rufescens* Swainson. Algae were collected monthly from July, 1975 to October, 1976 from a rocky intertidal area north of Dillon Beach, Marin County, California.

Egg capsules occur in the burrows of adults. These were carefully removed with forceps and placed in 9 cm diameter culture dishes containing 50 ml of filtered sea water.

Larval emergence from the capsules was unassisted. Cultures of 50 larvae per dish were maintained in refrigerated coolers at a salinity of 32‰ and at temperatures of 10°, 15° and 20° C. Water was changed every third day and

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larvae were fed a mixture of *Dunaliella tertiolecta* Butcher, *Phaeodactylum tricornutum* Bohlin, *Isochrysis galbana* Parke, and *Skeletonema costatum* (Greville). Concentrations of 55,000 algal cells/ml promoted satisfactory growth.

Live sperm were obtained from adult males crushed between a slide and coverslip. Sperm were then examined with oil immersion optics, utilizing Zeiss RA compound microscopes equipped with phase contrast and Nomarski phase interference. Other observations were made with a Wild M-5 stereomicroscope.

The size of larvae at specific larval stages was determined by averaging the measurements of 25 larvae at each stage. Line drawings of larvae were made from photographs taken with a Polaroid camera mounted on the Zeiss RA and follow the procedures outlined in Blake and Woodwick (1975).

Serial sections were made of 60 adult worms collected over a six month period from February to July, 1976. Worms were narcotized in 0.15% propylene phenoxylol (McKay and Hartzland, 1970) and fixed in Bouin's fluid. Sections were stained with Harris's hematoxylin and counterstained with eosin (Humason, 1972).

Larvae which were ready to metamorphose were offered various substrata in order to induce metamorphosis. Different substrata were tested by placing 20 larvae and the desired substratum in a small nytex container. The substrata tested included: sand, pieces of abalone shells and encrusting coralline algae, both with and without adults present. All containers were placed on the same water table. Each substratum was tested in this manner on two separate occasions.

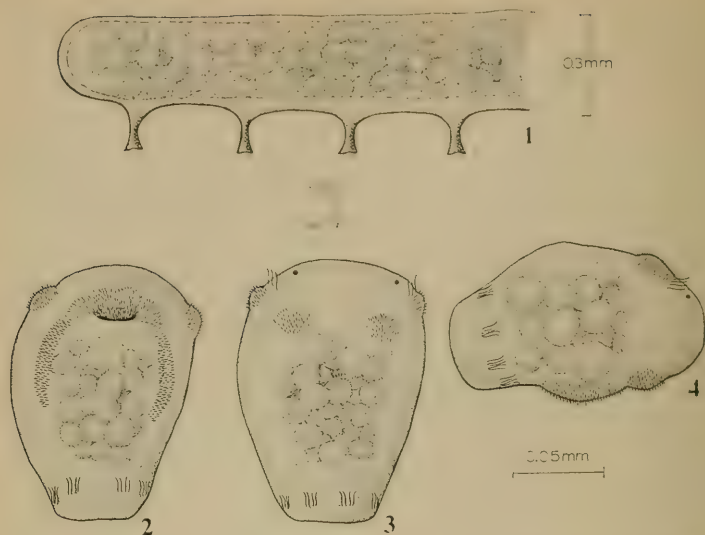
In order to determine if the larvae settled randomly on the surface of the encrusting algae or were attracted to a cue provided by previously settled worms, pieces of coralline algae containing *P. giardi* were collected in the field. Photographs of the surfaces of the algae were taken and enlarged. An index of non-randomness was prepared from measurements of the distances between burrow openings on the surfaces of the coralline algae (Pielou, 1974). These indices of non-randomness indicated if the burrows of settling worms were randomly distributed on the coralline algae or if the burrows were in some non-random pattern, which would suggest an influence of previously settled forms on the settling larvae.

RESULTS

Reproduction

The 60 adult worms serially sectioned included 5 sexually mature individuals, 3 females and 2 males. Mature individuals contained either eggs or sperm; there was no evidence of hermaphroditism. Gametes were limited to the medial segments of the body. The first epitokal segment ranged from segments 25 to 31 in females and from segments 27 to 29 in males.

Each epitokal segment of the female contained only three to six mature oöcytes. These coelomic oöcytes were convoluted, measured approximately 80 μ m in diameter and possessed a distinct nucleus with a large nucleolus. In addition, clumps of four to eight developing oöcytes were closely associated with the adult body wall, in epitokal segments.



FIGURES 1-4. *Polydora giardi* egg capsule (Fig 1); asetigerous larva, ventral view (Fig. 2); dorsal view (Fig. 3); lateral view (Fig. 4).

The coelom of epitokal segments of sexually mature males was filled with mature sperm. Sperm had the following average measurements ($N = 25$): total length, $42.7 \mu\text{m}$; acrosome, $1.85 \mu\text{m}$; nucleus, $5.7 \mu\text{m}$; middlepiece, $1.8 \mu\text{m}$ and tail, $33.3 \mu\text{m}$. Sperm were of the aberrant type (Franzen, 1956). Sperm transfer was not observed and the method of fertilization is unknown.

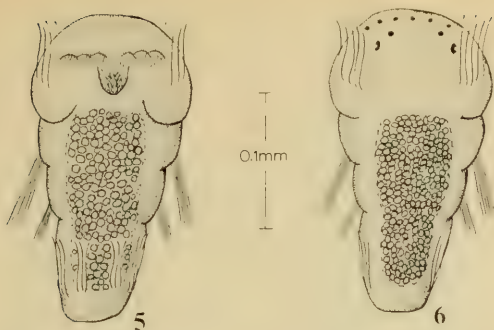
Egg capsules were found from February through November. The capsules are of an unusual structure, consisting of an elongated, hollow, transparent cylinder which is attached to the wall of the tube by regularly spaced, unpaired filaments (Fig. 1).

Cleaved embryos were spherical and measured $90 \mu\text{m}$ in diameter. All eggs were fertilized; there was no evidence of nurse cells.

Development in the capsule

Cleavage is spiral and unequal. Larvae remain in the capsules until the three-setiger stage, when they escape into the plankton. Development time from initial egg deposition to hatching takes approximately 8 days at 15°C .

Early asetigerous larvae (Figs. 2-4). After 4 days the embryos have differentiated into mobile larvae measuring $120 \mu\text{m}$ long and $95 \mu\text{m}$ wide. Large



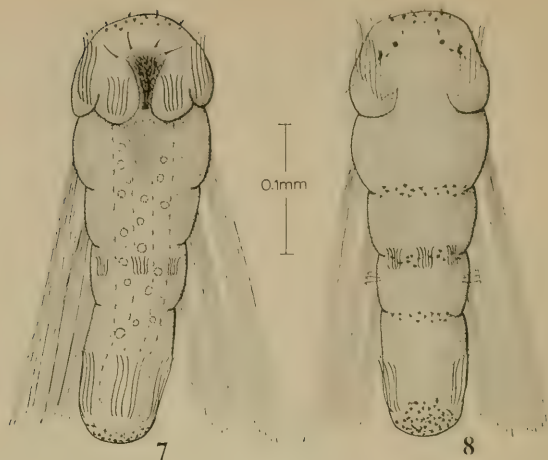
FIGURES 5-6. *Polydora giardi* encapsulated two-setiger larva, ventral view (Fig. 5); dorsal view (Fig. 6).

macromeres fill the central portion of the larva. Smaller oil droplets are concentrated in the anterior region; internal differentiation is not evident.

Two small, circular eye spots occur dorsally, anterior to the prototroch (Fig. 3). The prototroch consists of four dense patches of cilia; two dorsal and two lateral. A pre-prototroch is situated dorsally, slightly above the lateral prototrochal patches. The large, circular, ciliated vestibule dominates the ventral side of the larva (Fig. 2). The anterior half of the vestibule is surrounded by four ciliated knobs. The mouth is not developed at this stage. Posterior to the vestibule, two ventral ciliary ridges extend down the mid-section of the larva, appearing as a hump when viewed from the side (Fig. 4). The telotroch is formed by several patches of cilia arranged in an incomplete ring around the posterior portion of the larva.

Two-setiger larvae (Figs. 5, 6). With continued development, the larvae elongate and are characterized by a distinct prostomium and two setigerous segments. The larvae average $168\ \mu\text{m}$ long and $100\ \mu\text{m}$ wide. The first two segments bear short larval setae emerging from setal sacs, while the third segment is achaetous.

The head is wider than the body region. Cup-shaped eyes occur dorsally on each side of the head. Two circular eyes are anterior and medial to the cup-shaped pair. Six small, black pigment spots are arranged in an arc across the prostomium and anterior to the circular eyes. The pre-prototroch is lost and the prototroch is now well-developed, consisting of four patches of cilia. The four knobs surrounding the anterior portion of the vestibule have developed into six ciliated mounds which extend across the ventral surface of the prostomium. The vestibule is longer and deeper. Nototrochs, gastotrochs and grasping cilia are not present. The telotroch is well-developed, consisting of six patches of cilia nearly encircling the pygidium.



FIGURES 7-8. *Polydora giardi* pelagic three-setiger larva, ventral view (Fig. 7); dorsal view (Fig. 8).

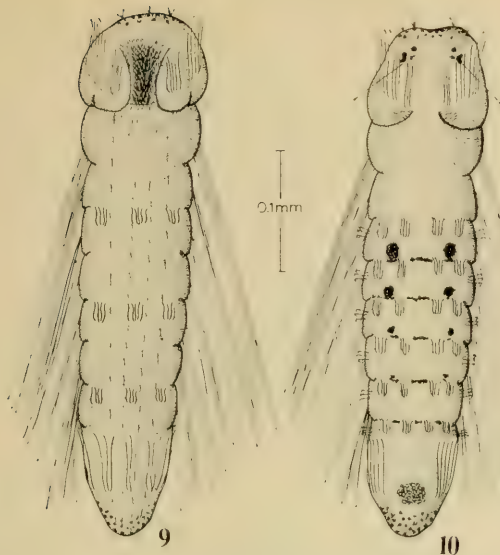
The oil droplets that were present earlier within the anterior portion of the larva are no longer seen. The intestine contains yolk and the internal differentiation of the intestinal walls is now apparent. The mouth and anus are not complete.

Development in the plankton

Larvae are released from the egg capsules at the three-setiger stage, are capable swimmers and are positively phototactic. In the laboratory, larvae are planktotrophic during a planktonic period which exceeds 25 days at 15° C.

Three-setiger larvae (Figs. 7, 8). This stage is reached in 8 days. Planktonic three-setiger larvae have a mean length of 240 μ m and a maximum width of 90 μ m at the head. Setal sacs of setiger 1 are large and conspicuous, containing seven to ten long, serrated setae. Fewer serrated setae emerge from the setal sacs of setigers 2 and 3. The heavily ciliated vestibule is surrounded anteriorly by four to five large tactile cilia (Fig. 7). The mouth is formed and the digestive tract is complete. Yolk is still apparent within the gut. Six eyes occur on the dorsal surface of the head; two large, lateral, cup-shaped eyes, and two pairs of smaller, medially located, round eye spots (Fig. 8). Two large, tactile cilia arise from a region slightly posterior to the cup-shaped eyes.

The prototroch is composed of six distinct patches of cilia, two medial ventral patches of cilia, two ventrolateral patches (Fig. 7) and two dorsolateral patches (Fig. 8). Two patches of inconspicuous nuchal cilia are present. A



FIGURES 9-10. *Polydora giardi* eight-setiger larva, ventral view (Fig. 9); dorsal view (Fig. 10).

broad neurotroch extends posteriorly from the vestibule to setiger 2. Nototrochs occur on setiger 3 which are slightly anterior to the long, laterally directed, grasping cilia. Gastrotrochs occur in three patches on setiger 3.

Pigment is sparse, with a few black granular spots scattered dorsally along the boundaries of each segment. A little pigment also occurs on the pygidium.

Eight-setiger larvae (Figs. 9, 10). The eight-setiger stage represents the mid-point in planktonic development. At 15° C, this stage is reached in approximately 12 days. The mean size of the eight-setiger larva is 504 μ m long and 168 μ m wide. Setal sacs of setiger 1 remain large with respect to those of other segments, and their contained setae are the longest.

Significant changes in ciliation have occurred. The neurotroch now extends midway into setiger 2 (Fig. 9). The prototroch and telotroch are formed by eight and five groups of cilia, respectively. Nototrochs and grasping cilia occur on setigers 3 to 8. Gastrotrochs occur on setigers 3, 5 and 7.

The greatest change in morphology is in amount and position of pigment. The dorsal pigment is now more pronounced and coalesced into prominent black spots on setigers 3-5 (Fig. 10). Intersegmental pigment remains on setigers 3-8. Rust-colored pigment occurs within the pharynx and a golden pigment

occurs in the dorsal gap of the telotroch. Ventrally, small amounts of black pigment occur on both the prostomium and pygidium.

In laboratory specimens, the gut is bright green from ingested algae. Embryonic yolk is no longer present.

Seventeen-setiger larvae (Fig. 11). The 17-setiger stage represents the fully developed planktonic larva and the final phase preceding metamorphosis. These larvae are 980 μm long and 180 μm wide. Palps are short and inconspicuous.

Nototrochs and grasping cilia begin on setiger 3 and continue on each succeeding segment. Each nototroch consists of four patches of cilia. Gastrotrochs, consisting of four patches of cilia, occur on setigers 3, 5, 7, 10, and 13.

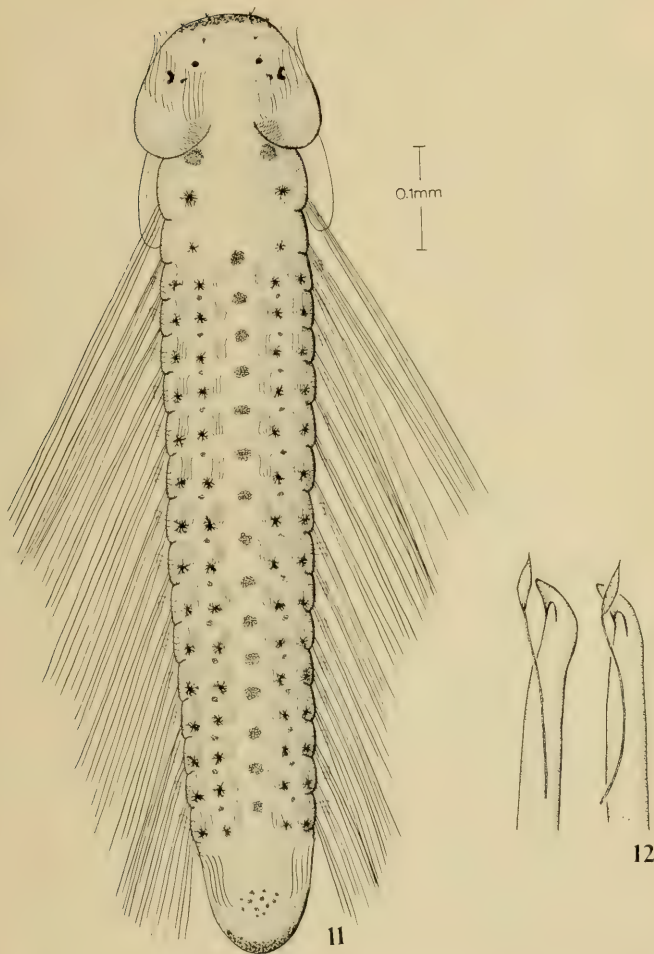
The dorsal pigment pattern is now fully developed and diagnostic for *P. giardi*. Two golden pigment spots are present on either side of setiger 1. Two small, black, lateral chromatophores occur on setigers 1 and 2. These are replaced in subsequent setigers by four black chromatophores in a line across each segment. A large medial cluster of black-pigmented cells begins on the posterior margin of setiger 2; from setiger 3, two smaller groups of pigmented cells occur lateral to the larger cluster. A small, black pigment spot, not visible dorsally, is present on the lateral anterior edges of each segment. Black pigment also occurs on the pygidium. Pigment is absent from the ventral surface. Rust-colored pigment occurs in the pharyngeal area.

The modified spines and accompanying companion setae of setiger 5, characteristic of the adult, are developed but not emergent. Each spine bears a small accessory tooth (Fig. 12).

Settling and metamorphosis. The larvae of *P. giardi* were not easily induced to metamorphose. Of the various substrata, including sand, abalone shells and coralline algae, offered to 200 larvae, only 2 metamorphosed. Both larvae metamorphosed in the presence of coralline algae. The ability of larvae to delay metamorphosis was considerable. One planktonic larva was kept in the laboratory in excess of 5 months without metamorphosing. There was no apparent change in size or morphology of this larva.

The values of Pielou's index calculated from the photographs of burrow patterns on algal surfaces indicate that the larvae settle and metamorphose randomly on the surface of the encrusting algae. Thus, the settling larvae do not appear to be attracted by other established *P. giardi*.

Morphological changes at metamorphosis include loss of the prototroch, telotroch, gastrotrochs, nototrochs, neurotroch, nuchal cilia and serrated larval setae. The benthic juvenile secretes a mucoid tube and begins to burrow into the calcareous substratum. Eyes that were present in the larva remain for a short time but are lost with continued growth. Palps, previously short and directed back along the body during planktonic development, are now elongated and project forward. The juvenile lacks pigment. Branchiae begin on setiger 9 and continue to setiger 19. The modified spines of setiger 5 emerge from the body wall and are accompanied by slender bilimbate setae (Fig. 12). The benthic juvenile resembles the adult worm in all but one characteristic: the size and shape of the pygidium. In the adult the pygidium is a thick, collar-like cuff that is longer than it is wide; in the benthic juvenile it is also a collar-like cuff, but it is not as long as it is wide.



FIGURES 11-12. *Polydora giardi* pelagic 17-setiger larva, dorsal view (Fig. 11); larval setae of fifth setiger of 17-setiger larva (Fig. 12).

DISCUSSION

Mesnil (1896) suggested that *Polydora giardi* was a simultaneous hermaphrodite. He found that 90% of the individuals collected during the summer months in France contained mature sperm in the coelom, one-third of which also contained mature oöcytes. Oöcytes occurred in the first 18 segments and numbered two to five oöcytes per segment. Sperm were never found anterior to setiger 9. Our observations suggest that the specimens of *Polydora giardi* found in California are dioecious. Another apparent difference between the California and French populations is the number of individuals found to be sexually mature. During the months of July and August, Mesnil found 90% of the adults to be sexually mature, while we found only 8.3% of the worms collected over a 6-month period from February to July to be sexually mature. Mesnil does not indicate if the 90% figure was maintained throughout the year or only for a short period of time in late summer. This situation contrasts with the northern California population, which appears to have fewer sexually mature individuals at any one period but maintains a few sexually productive individuals throughout most of the year, as evidenced by egg capsules found in female burrows from February through November. These differences in reproduction between the California and European populations suggest that different species are involved. A comparison of adult specimens of *P. giardi* from California and Britain (provided by the British Museum), however, has demonstrated that they have the same morphology.

Blake (1969) recognized three types of egg capsules within species of the *Polydora*-complex. The most common type includes small capsules joined bead-like in a string, with each individual capsule attached to the wall of the adult tube by either one or two stalks. A second type of capsule occurs singly, attached to the tube by two thin extensions, but separate from its neighbor. The third type of capsule is a hollow cylinder filled with eggs, and attached to the burrow by extensions throughout its length. This last type of capsule was only known previously for *Polydora quadrilobata* Jacobi (Blake, 1969). *Polydora giardi* is the second species reported to have such a capsule.

The manner of deposition of spionid egg capsules was postulated by Söderström (1920). His theory states that capsular material is secreted by cells of the nephridial pores. The eggs are squeezed from the coelom through the nephridial tubes and are deposited in the capsule. This hypothesis has been confirmed for *Polydora ligni* Webster by Rice and Reish (1976). *Polydora ligni* has small individual capsules attached by two filaments to the inside walls of the burrows of adult females. The method of capsular formation and deposition which results in the long cylindrical capsules of *P. giardi* and *P. quadrilobata* is not known. Further studies are needed to determine the differences in the manner of egg capsule formation within the *Polydora*-complex.

The early encapsulated larvae of *Polydora giardi* closely resemble those of *Polydora aggregata* Blake and *Polydora socialis* (Schmarda) as described by Blake (1969). The last two species have ciliary patches in the area where the first setal sac will develop, while *P. giardi* has two large, ventrally located, ciliated ridges which extend posteriorly from the lower portion of the mouth.

These ridges are considered analogous to the ciliary patches first described by Wilson (1928) for *Polydora ciliata* (Johnston) and *Polydora hoplura* Claparède; it has been suggested that these ciliary patches provide a means of movement in the egg capsule prior to the development of ciliary bands (Blake, 1969).

Planktonic larvae of *P. giardi* closely resemble the larvae of *Polydora concharum* Verrill, as described by Blake (1969), and *Polydora flava* Claparède and *Polydora caeca* (Oersted), as described by Hamnerz (1956). All four species have similar larval morphologies, which include a long slender body form, short inconspicuous palps, lack of branchiae before metamorphosis and similar dorsal pigment patterns. *Polydora concharum* and *P. giardi* lack ciliated pits posterior to the mouth, while *P. caeca* and *P. flava* have simple ciliated pits. *Polydora giardi* and *P. flava* have gastrotrochs on setigers 3, 5, 7, 10, and 13; *P. caeca* on 3, 5, 7, and 9 and *P. concharum* on 3, 5, and 7. All four of these species burrow into calcareous substrata and are commonly found in encrusting coralline algae. Hamnerz (1956) and Blake (1969) both suggest that larval studies are useful in relating species. The similarities of the larval morphologies of these four species are paralleled in their adult morphologies.

The developmental rate of *P. giardi* is inversely related to temperature. An increase in temperature decreases developmental time. At 10° C, the larvae acquired 17 setigers in 32 days, while at 20° C it took only 20 days to reach the same stage.

Many pelagic larvae settle in response to the presence of the adult; however, *P. giardi* appears to be attracted by the encrusting algae. This conclusion is supported by the values of Pielou's index of non-randomness, which indicate that the larvae settle randomly on the surface of calcareous substrata. One would expect a non-random settling pattern if the larvae were attached to a cue supplied by the adults. Further support for the conclusion that larvae are attracted by the algae and not the adults comes from the fact that the two larvae that did metamorphose in the laboratory did so in the containers containing coralline algae free from adult worms or their tubes.

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SUMMARY

The larval development of *Polydora giardi* is described. Adults occur in burrows found in coralline algae and abalone shells. Sexes are separate. Females deposit eggs in hollow, cylindrical capsules attached to the wall of the tube by filaments. Larvae are released from capsules at the three-setiger stage. Planktonic development exceeds 25 days at 15° C. Larvae settle randomly on the surface of calcareous substrata and metamorphose in the presence of coralline algae. Only 8.3% of the population were sexually mature over a 6-month period

from February through July. The larvae of *P. giardi* closely resemble those of *P. flava*, *P. cacca*, and *P. concharum*. This larval resemblance is also reflected in adult morphology.

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PUPAL DIAPAUSE IN TROPICAL FLESH FLIES: ENVIRONMENTAL AND ENDOCRINE REGULATION, METABOLIC RATE AND GENETIC SELECTION

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Periods of dormancy occupy a conspicuous component in the life history of most temperate region insects. Winter is a very predictable event, and daylength serves as a reliable seasonal indicator. But, on the equator, daylength is constant throughout the year, the coldest month is only a few degrees colder than the warmest, and the rainfall pattern can be extremely variable from year to year. Do insects have a diapause under these circumstances, and if so, how is the diapause regulated? Information for tropical insects remains scant, but as more species are examined, it is becoming apparent that periods of developmental arrest may be important for many tropical species (Nishida, 1955; Van Dinther, 1961; Norris, 1965; Entwistle, 1968; Omer and Cloudsley-Thompson, 1968; Usua, 1973; Denlinger, 1974; Scheltes, 1976).

The present study is based on flesh flies (Diptera: Sarcophagidae), a widespread group of flies whose larvae feed on carrion, open wounds, and feces, or parasitize other arthropods. In temperate zones, flesh flies enter an over-wintering pupal diapause in response to the short days of late summer (Fraenkel and Hsiao, 1968a; Denlinger, 1971b, 1972a; Saunders, 1971; Roberts and Warren, 1975; Vinogradova, 1976) and nine months may elapse before an adult emerges from the puparium (Denlinger, 1972b). In East Africa, flesh flies enter pupal diapause in response to cool temperature: diapause can readily be induced by low temperatures in the laboratory (Denlinger, 1974), a few diapausing pupae have been observed in the field during the cold months of July and August (Denlinger, 1978), and trapping records show a sharp drop in number of adult flies at the end of August (Denlinger, in preparation). This study investigates diapause in several species of flesh flies from Africa and South America in an attempt to gain an overview of the physiologic mechanisms of dormancy in a tropical environment.

MATERIALS AND METHODS

Experimental flies

Fly colonies originated from wild-caught females collected from four localities in East Africa and one locality in South America (Table I). Stock cultures were maintained in a controlled environment room at $25 \pm 1^\circ \text{C}$ with a 12:12 light:dark cycle using the rearing method described by Denlinger (1972a). Environmental conditions for the experimental flies were provided by environ-

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mental chambers (Percival Refrigeration Company and Sherer-Gillett Company) that controlled temperatures within 0.5°C and provided programmed illumination. All experiments were completed within two years after collecting the wild females.

Criteria for diapause

Pupae were considered to be in diapause if they remained as undifferentiated phaenerocephalic pupae when their siblings had either emerged as adults or were pharate adults with the pigmented eyes and black bristles (Fraenkel and Hsiao, 1968b) characteristic of an advanced stage of adult differentiation. Arrest of development was never observed in any stage other than the pupa.

Hormonal applications

Crystalline ecdysterone obtained from Dr. Jizba of the Czechoslovak Academy of Sciences was dissolved in 96% ethanol and diluted to 10%. Finely-drawn glass capillaries were calibrated and used to inject ecdysterone solutions of 0.5 or $1\text{ }\mu\text{l}$ into the neck region of pupae, from which approximately $1\text{ }\mu\text{l}$ of hemolymph had been bled. The anterior caps of the puparia had previously been removed to gain access to the pupae. Following injection, pupae were attached to double-stick tape on a filter paper disc and placed in Petri dishes.

The juvenile hormone analogue isopropyl (2E, 4E)-11-methoxy-3, 7, 11-trimethyl-2, 4-dodecadienoate (ZR515, Altosid[®]) was applied directly to the heads of diapausing pupae. By distributing 0.5 mg of the analogue among 10 pupae, a dose of approximately $50\text{ }\mu\text{g}$ was received by each individual. Solvents could not be used as carriers, since acetone and a number of related solvents, by themselves, are quite effective in breaking diapause (Ždárek and Denlinger, 1975).

Measurement of O_2 consumption

Individual pupae were placed in separate vessels of a Scholander respirometer (Mark Company, Brockton, Mass.) containing a folded strip of Whatman No. 40 filter paper moistened with 0.3 ml 10% KOH to absorb CO_2 . Pupae were equilibrated for 2 hr before recording the initial reading, and thereafter readings were taken at 24 hr intervals, as described by Denlinger, Willis and Fraenkel (1972). A temperature of 15°C was obtained by operating the heating water bath of the respirometer within a cold room provided with 12 hr of daily illumination.

RESULTS

Daylength

The slight seasonal variation in daylength observed near the equator does not appear to be involved in the induction of flesh fly diapause. It was not possible to demonstrate an effect of photoperiod at temperatures of 12° , 15° , 18° , 25° , and 30°C . Larvae of *Sarcophaga inzi*, *S. par*, *Pocillometopa spilogaster* and *P. punctipennis* were tested throughout the temperature range at daylengths of

TABLE I

Influence of larval temperature on induction of pupal diapause in species of flesh flies (Sarcophaga and Pöcilonetopa) from five localities in East Africa and South America.

Locality	Species	Incidence of diapause at various temperatures									
		12° C		15° C		18° C		25° C		30° C	
		N	% diapause	N	% diapause	N	% diapause	N	% diapause	N	% diapause
Nairobi, Kenya (1° 18' S, 1700 m)	<i>S. par</i>	2554	7	575	0	306	0	4237	0	82	0
	<i>S. irzi</i>	723	31	3215	46	1515	0	8113	0	730	0
	<i>S. exuberans</i>	280	48	519	71	—	—	1085	0	—	—
	<i>P. spilogaster</i>	1151	79	3752	56	1194	12	5688	0	151	0
	<i>P. punctipennis</i>	732	54	1525	12	511	0	4827	0	298	0
Malaya, Tanzania (8° 54' S, 1735 m)	<i>S. par</i>	5361	18	1144	0.4	—	—	6908	0	—	—
Addis Ababa, Ethiopia (9° 2' N, 2440 m)	<i>S. monospila</i>	256	60	161	0.6	105	0	1911	0	—	—
Mombasa, Kenya (4° 4' S, 15 m)	<i>S. haemorrhoidalis</i>	—	—	19	42	103	9	728	0	—	—
Belém, Brazil (1° 27' S, 13 m)	<i>S. ruficornis</i>	1825	98	780	99	3690	38	1041	0	410	0

11, 12, 13, and 16 hr, and with *S. inzi*, *P. spilogaster* and *P. punctipennis* from Nairobi and *S. par* from Mbeya, daylengths of 11.25, 11.5, and 12.5 hr were also tested at 12° and 15° C. Even flies from Mbeya, which normally experience a seasonal difference of about an hour between the longest and shortest days, did not respond to daylength in laboratory experiments.

Temperature

All flies tested entered pupal diapause in response to cool temperature. As shown in Table I, 18° C was the highest temperature at which diapause was observed. The incidence of diapause increased at lower temperatures. Since an effect of daylength could not be established, data in Table I were pooled from experiments performed at photoperiods ranging from 11 to 16 hr of daylight. As indicated by Denlinger (1974), the temperature on each day of larval life is important, and the diapause can not be induced by merely exposing fully grown larvae or young pupae to cold temperature. Thus, the dormancy observed is not an immediate response by the pupa to low temperature, but a response programmed during an earlier developmental stage.

Pupae that entered diapause eventually reinitiated development spontaneously under constant temperature conditions. The lower the temperature, the longer was the diapause. For example, mean $\pm$ s.e. duration of diapause in *P. spilogaster* was 72.6 ± 2.9 days at 18° C ($N = 108$), 100.9 ± 2.2 days at 15° C ($N = 268$) and 234.6 ± 6.8 days at 12° C ($N = 207$).

Adult development can be initiated in pupae held at 12° C by a four-day exposure to 25° C (Denlinger, 1974). Further experiments with *P. spilogaster* show that the 4 days at 25° C need not occur as a single block of 96 hr but instead smaller units of time at 25° C can be summed over a period of several weeks (Table II). Pupae that had been in diapause for one month at 12° C were transferred to 25° for various periods and then returned to 12° C. Although one day at 25° C was not sufficient for diapause termination, pupae that were given this treatment each week for 4 weeks broke diapause as readily as pupae that received a single block of four days at 25° C. For each of the time units shown in Table II, the summation of subcritical "doses" of 25° C closely paralleled the effect from a single exposure of the same total duration. In the Brazilian fly, *S. ruficornis*, even shorter periods at 25° C were effective in initiating development. Exposure to 25° for 12 hr stimulated development in 45% of the pupae ($N = 20$) and 24 hr at the warmer temperature was sufficient for initiating development in 75% of the pupae ($N = 20$). The accumulation of subcritical amounts of high temperature effects was also apparent: a 3 hour period by itself was ineffective, but three-hour daily exposure for four days (12 hr cumulative) prompted development in 54% ($N = 24$) of the pupae and three-hour daily exposure for eight days (24 hr cumulative) initiated development in 77% of the pupae.

Thermoperiod

Cool daytime temperatures produced both a higher incidence of diapause and longer diapause than cool night temperatures (Table III). In experiments A to

TABLE II

Summation of subcritical exposures to 25° C in diapause pupae of *P. spilogaster*. Pupae were held at 12° for 1 month, transferred to 25° for various periods and then returned to 12° C.

Number of days at 25° C on various weeks					Cumulative time at 25° C (days)	No. of pupae	% breaking diapause
Week 1	Week 2	Week 3	Week 4	Week 5			
0	0	0	0	0	0	25	0
1	0	0	0	0	1	25	8
2	0	0	0	0	2	23	22
1	1	0	0	0	2	20	38
3	0	0	0	0	3	21	48
1	1	1	0	0	3	21	57
4	0	0	0	0	4	20	85
1	1	1	1	0	4	20	90
5	0	0	0	0	5	25	96
1	1	1	1	1	5	19	89

E, *S. ruficornis* was reared with a daily cycle of 12 hr at 10° and 12 hr at 17° C; half of the larvae in each batch received 10° C during the photophase and half received 10° C during the scotophase. To minimize error from any undetected temperature differences in the two environmental chambers, photoperiods in the two chambers were reversed after experiments A to C. Thus, in experiments D and E the previous temperature settings were used, but the photoperiods were

TABLE III

Thermoperiodic effects on pupal diapause induction and diapause duration in *S. ruficornis* reared with a daily cycle of 12 hr light and 12 hr dark.

Experiment	Day temp.	Night temp.	No. of pupae	% diapause	Diapause duration (mean days $\pm$ s.e.)
A	10° C	17° C	256	100	80.6 $\pm$ 3.6
	17	10	316	85	43.6 $\pm$ 1.6
B	10	17	127	94	73.4 $\pm$ 2.9
	17	10	183	57	42.3 $\pm$ 2.7
C	10	17	137	100	102.5 $\pm$ 4.9
	17	10	183	90	47.0 $\pm$ 2.0
D	10	17	181	97	77.4 $\pm$ 3.2
	17	10	175	86	44.3 $\pm$ 2.0
E	10	17	109	95	—
	17	10	128	72	—
F	10	20	197	36	—
	20	10	215	5	—
G	10	20	1356	29	—
	20	10	982	7	—

reversed. Consistently higher levels of diapause occurred when the colder temperature coincided with the photophase. The incidence of diapause using combined data from replications A to E was 97.6% ($N = 810$) for daytime exposure to 10° C and 79.2% ($N = 985$) for nighttime exposure to 10° C (Pearson $\chi^2 = 138.5$, $df = 1$, $P < 0.01$). With fluctuations between 10° and 20° C (experiments F and G), the higher incidence of diapause likewise coincided with cold daytime temperature. A constant temperature of 15° C, however, produced a higher level of diapause (Table I) than was observed with daily thermoperiods of 10° and 20° C (Table III).

Duration of diapause was also influenced by the temperature cycle. As shown in Table III, pupae that received 10° during the photophase remained in diapause for a significantly longer period than their siblings that received 10° C during the scotophase (Student's t -test, $P < 0.01$ for experiments A-D).

Species and geographic variability

Although flies from all of the stock colonies entered diapause in response to cool temperatures, species and locality differences in the temperature reaction are apparent in Table I. Threshold for diapause was not uniform even among the various species from a single locality such as Nairobi, but a comparison of data from the five different localities suggested that the diapause threshold may be higher in warmer environments. Belém and Mombasa are much warmer than Nairobi, whereas both Mbeya and Addis Ababa are slightly cooler than Nairobi. In flies from the coldest regions, Mbeya and Addis Ababa, the diapause threshold was around 12° C, whereas flies from warm Belém or Mombasa readily entered diapause at 15° or 18° C. The threshold for Nairobi flies appeared to be intermediate.

Water content of larval food

A minor effect of water was demonstrated in the programming of diapause. A 10% increase in water content of the larval food (homogenized beef liver) elevated the incidence of diapause 10% at 15° C. Conversely, when 10% of the moisture content of the liver was removed in a drying oven, the incidence of diapause declined 18%. Experiments were performed on *P. spilogaster*: 58.7% ($N = 230$) of the flies entered diapause with normal liver, 69.3% ($N = 339$) with "wet" liver (Pearson $\chi^2 = 28.4$, $df = 1$, $P < 0.01$), and 41.0% ($N = 407$) with "dry" liver (Pearson $\chi^2 = 18.5$, $df = 1$, $P < 0.01$). Water content was capable only of modifying the temperature effect on diapause induction and could not elicit diapause unaccompanied by cool temperature. No effect on diapause could be detected when adults, wandering larvae, or pupae were exposed to relative humidities ranging from 20 to 92%.

Sex ratio

Sex was determined for diapausing individuals of *P. spilogaster* that originated from batches with a diapause incidence less than 20% (reared at 18° C). Males and females entered diapause in nearly equal proportion. The observed ratio

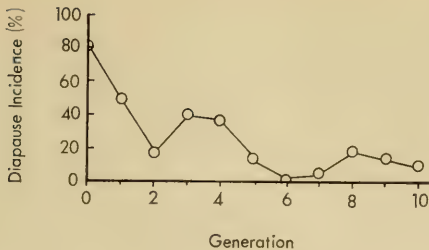


FIGURE 1. Genetic selection for non-diapausing pupae of *P. spilogaster* at 15° C. Each point represents 244 to 326 pupae; 100 non-diapausing flies were selected for propagation in each generation.

of 1.03 ♂ : 1♀ (N = 167) at a low incidence of diapause suggests that the skewed sex ratio favoring males for diapause in the temperate region (Denlinger, 1972a) does not operate among the tropical species.

Genetic selection for non-diapause

The incidence of diapause was readily lowered by selection for non-diapause individuals. Starting with a parental generation of *P. spilogaster* reared as larvae at 15° C, flies that did not diapause were selected for propagation. All subsequent generations of larvae were reared in the same environmental chamber at 15° C with a 12-hr daily photophase. As shown in Figure 1, selection against diapause was rapid. The level of diapause dropped from 80 to 50% in the first generation and remained less than 20% after the fifth generation.

Oxygen consumption

Oxygen consumption of individual pupae of *S. inzi* and *P. spilogaster* was monitored at 15° C for a 38-day period beginning 2 weeks after the onset of diapause. Daily records of four representative individuals (Fig. 2) show that the rate of O₂ uptake during diapause was not constant. Days of high O₂ consumption (30–80 μl/g hr) were interspersed between days in which O₂ consumption was non-detectable. Mean ± s.e. periodicity of the metabolic cycles was 4.3 ± 0.3 days (N = 35 cycles observed) for *S. inzi* and 5.3 ± 0.8 days (N = 23 cycles observed) for *P. spilogaster*, but in comparison to similar cycles observed in diapausing pupae of temperate region flesh flies (Denlinger *et al.*, 1972), the cycles in the tropical flies were considerably more irregular. In flies that did not diapause, the QO₂ followed the smooth U-shaped curve characteristic of metamorphosis in holometabolous insects. In data not shown, the nadir of the U-curve at 15° C was 60 μl O₂/g hr for non-diapause individuals of *S. inzi* and 75 μl O₂ for *P. spilogaster*. Thus, the QO₂ peaks in diapause pupae usually did not exceed the lowest metabolic rate observed in non-diapause pupae. Mean diapause QO₂ in *S. inzi* calculated from measurements of five pupae over a 38-

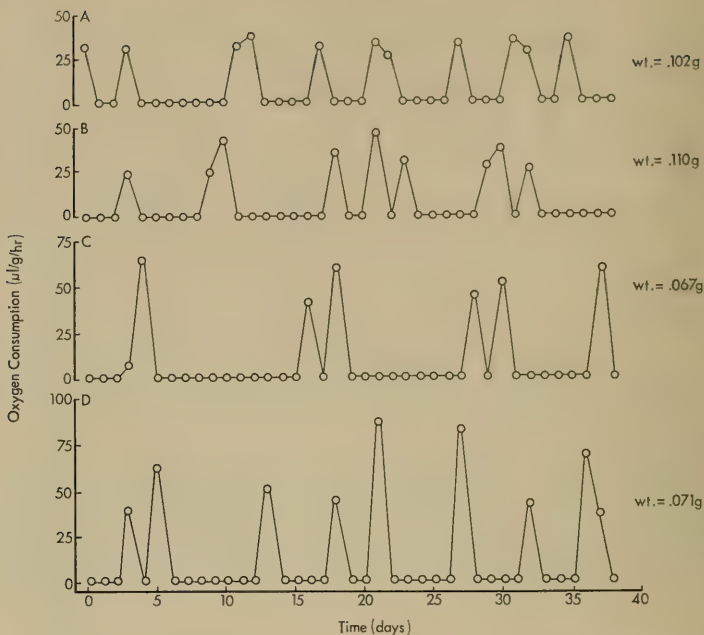


FIGURE 2. Infradian cycles of oxygen consumption in individual diapausing pupae of *S. inzi* (A and B) and *P. spilogaster* (C and D) at 15° C.

day period was $7.9 \mu\text{l O}_2/\text{g hr}$ and in five pupae of *P. spilogaster* the mean QO_2 was $10.9 \mu\text{l O}_2/\text{g/hr}$, rates that are 13–15% of the minimum rate observed in non-diapause individuals at the same temperature.

Endocrine involvement

Pupae of *S. inzi* that had been in diapause at 15° C for 2 weeks were injected with 10% ethanol or solutions of ecdysterone in 10% ethanol. After 20 additional days at 15° C, pupae were examined for diapause termination. Development was initiated in 44% of the pupae with $5 \mu\text{g}$ ecdysterone (Fig. 3).

By itself, topical application of juvenile hormone analogue ($50 \mu\text{g}$ ZR515/pupa) broke diapause in only 8% of the pupae, but when applied in concert with ecdysterone injections, juvenile hormone analogue greatly boosted the rate of diapause termination (Fig. 3). The effective dose of ecdysterone required for 40% diapause termination was reduced from 5 to $0.05 \mu\text{g}$ by the addition of

juvenile hormone analogue. In a similar experiment with *P. spilogaster*, 5 μ g ecdysterone stimulated development in 52% of the pupae ($N = 25$) and the addition of juvenile hormone analogue increased the response to 80% ($N = 25$).

In flies from the temperate region, diapause can be broken readily with a topical application of acetone or several other organic compounds (Ždárek and Denlinger, 1975). These agents, ineffective on head-ligated pupae, apparently trigger development by activating the brain or ring gland. Various chemicals were tested topically (5 μ l) on groups of 20 diapause pupae of *P. spilogaster* held at 15° C. After 20 days, no development was observed in pupae receiving a topical application of water or 96% ethanol, but the following agents promptly stimulated adult development: acetone (65% diapause termination), diethyl ether (100%), methyl ethyl ketone (100%), dichloromethane (90%), *n*-pentane (100%), *n*-hexane (80%), acetaldehyde (25%), and benzene (90%). Toluene and ethyl acetate were moderately effective but survivorship was low. Dimethyl sulphoxide, benzaldehyde and benzyl alcohol were highly toxic and killed all the pupae within the test period.

DISCUSSION

Among flesh flies, reliance upon temperature as the major cue for diapause induction is unique to tropical species. The photoperiodic cues used extensively by their temperate region relatives in North America (Fraenkel and Hsiao, 1968a; Denlinger, 1971b, 1972a), Europe (Saunders, 1971; Vinogradova, 1976), Japan (Kurahashi and Ohtaki, 1977) and Australia (Roberts and Warren, 1975) do not appear to contribute to diapause programming in the African and South American flies living within 10° of the equator. By temperate region standards, the annual variation in temperature appears very slight. During a 29-month period in Nairobi, the range of mean weekly temperatures was only 6° C (Denlinger, 1978). But, the seasonal temperature change is a very predictable component of the environment, and perhaps offers a more reliable seasonal cue for the flies than rainfall pattern. Although amount of rainfall differs conspicuously among seasons in East Africa, the timing can be extremely erratic, and,

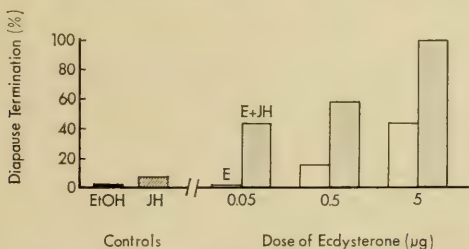


FIGURE 3. Diapause termination in pupae of *S. inzi* injected with various doses of ecdysterone in 10% ethanol or treated with a combination of ecdysterone and a 50 μ g topical application of a juvenile hormone analogue. Each bar represents 25 pupae.

for insects such as flesh flies, which are not immediately dependent upon plants for food, the direct impact of the rains may be relatively minor. During the coldest months in Nairobi, July and August, mean weekly temperatures of 16° to 17° C and mean weekly minima around 10° C are normal. Mean temperatures at this time of year are thus near the laboratory threshold for diapause induction, and field experiments (Denlinger, 1978) have shown that a few individuals do indeed enter pupal diapause in Nairobi during the cold days of July and August. In Malaise trap samples, adult flesh flies are well represented from May to August, but their numbers drops sharply in September (Denlinger, in preparation), again suggesting that a portion of the pupae may be slumped toward diapause in July and August.

It appears that the threshold for diapause is at the fringe of ecologically relevant temperatures in each locality studied. Thus, flies from the warmest areas, Mombasa and Belém, entered diapause at 18° C, whereas flies from the coldest areas, Addis Ababa and Mbeya, entered diapause only at 12° C.

Daytime temperature is apparently more crucial for determining diapause than night temperature. In laboratory experiments using different temperatures for the photophase and scotophase, cool photophase temperatures consistently yielded a higher incidence of diapause than cool scotophase temperatures. Thermoperiodism plays an important role in several insects from the temperate region, but normally a cool scotophase, rather than photophase, is more conducive for diapause (Menaker and Gross, 1965; Beck, 1968, 1977; Chippendale, Reddy and Catt, 1976; Greenfield and Karandinos, 1976). The distinct reversal of this trend among the tropical flesh flies should facilitate the fly's ability to accurately determine the coldest season of the year. Night temperatures in February (13.4° C mean monthly minimum) are nearly as low as night temperatures in July (11.5° C), but in February daily temperature range is great (14.6° C mean monthly range vs. 10.8° C in July) and day temperatures (28.0° C mean monthly maximum) are routinely 5 or 6 degrees above July day temperatures (22.3° C) (Anonymous, 1967). Thus, cool day temperatures most accurately identify July and August as the coldest months.

A strong genetic component is involved in maintaining the fly's response to environmental temperature. When non-diapause individuals were selected, the level of diapause at 15° C dropped from 80% in the parental generation of *P. spilogaster* to 10% in the tenth generation. Thus, genetic selection for an environmental response, well known in many temperate region species (Danilevskii, 1965; Beck, 1968; Hoy, 1977; Kurahashi and Ohtaki, 1977), can swiftly alter the response profile of a population and permit a diapause response finely tuned to the local environment.

It is quite possible that cold temperature may not be the "adverse" factor in the environment that has channeled the evolution of diapause, but instead flies may be using cold temperature as a token to signal another adversity. Our knowledge of their biology is sketchy, but flesh flies apparently can utilize carcasses, mammalian wounds and animal feces, and parasitize a broad spectrum of insects and other arthropods (Aldrich, 1916). Seasonal availability of suitable food resources and close packing of competitors in the tropics (MacArthur, 1972),

rather than an unfavorable temperature, may provide the selective advantage for diapause.

Considering the dramatic role of rainfall in determining tropical seasons, it seemed likely that moisture levels in the food or relative humidity might exert a major impact on the programming of diapause among flesh flies. Such was not the case. The only effect observed was a slight, but consistent, elevation of diapause with increased moisture content of the larval medium. Above the temperature threshold for diapause, the effect was not observed. In the field, the moisture effect should enhance the effect of low temperature during July and August. The extensive cloud cover and low temperature at this time of year (Anonymous, 1967) greatly retard desiccation in food resources such as animal carcasses or feces. At other times of the year, even during the rainy seasons, temperatures are higher, hours of bright sunshine are greater (*e.g.*, 9.5 hr bright sunshine/day in February *vs.* 4.2 hr in August), and desiccation occurs more rapidly. A diapause-enhancing effect of moisture is also seen in flesh flies from the temperate region (Denlinger, 1972a), but for several other insects the opposite is true. Decreased moisture content increases the incidence of diapause in *Lucilia sericata* (Mellanby, 1938), *Busscolu fusca* (Usua, 1973), and *Pectinophora gossypiella* (Raina and Bell, 1974).

Males and females of *P. spilogaster*, a fly from Nairobi, enter diapause in approximately equal proportion. This is in marked contrast to *S. bullata*, a North American species that has a highly skewed, male-favored, sex ratio for the diapause segment of the population (Denlinger, 1972a, 1972b). Although we cannot yet identify the selective advantage of the skewed sex ratio in the temperate region, apparently the same type of selective pressure is not being exerted on the tropical flies.

The duration of diapause appears to depend primarily upon temperature. Again, thermoperiodism appears to play a role. Cool daytime temperatures produce pupae that remain in diapause over twice as long as pupae exposed to the same cool temperature during the scotophase. This result was particularly surprising, since the pupa is normally encased within a puparium and buried several centimeters under the soil, where photoperiodic cues would, at best, be quite subtle. It is possible that diapause duration may have been programmed earlier during the larval stage. Thus, the results do not immediately imply pupal sensitivity to photoperiod.

Although diapause will persist for several months at the cool temperatures used for induction of diapause, initiation of adult development is very rapid at higher temperatures. Unlike the temperate region flies that remain in diapause for months at 25° C (Denlinger, 1972a), the tropical species *P. spilogaster* initiates development after only four days at 25° C. The 4 days at 25° C may be offered as a single block of time or as a series of smaller periods at weekly intervals. The net effect is the same: once the pupa has accumulated the equivalent of 4 days at 25° C, adult development will ensue. This type of temperature summation suggests a model in which high temperature leads to accumulation of a non-decaying product that either triggers development or gradually erodes a developmental inhibition.

Despite major differences in environmental factors that regulate diapause in the temperate and tropical regions, the neuroendocrine basis for diapause appears to be the same. Diapausing pupae of tropical flies initiate adult development in response to an injection of ecdysterone, as do their temperate zone relatives (Fraenkel and Hsiao, 1968b; Žďárek and Denlinger, 1975; Gibbs, 1976). Since the synthesis and release of ecdysone in flesh flies is regulated by the prothoracicotropic neurosecretion from the brain (Žďárek and Fraenkel, 1971), it seems likely that diapause is a result of the "shut-down" of the brain-prothoracic gland axis as in Williams's (1946) classic scheme for pupal diapause in Saturniids.

Two further results indicate a similar endocrine mechanism in the flies from the two regions: a juvenile hormone analogue enhances the ecdysone effect in both cases and the diapause of both regions can be terminated readily by topically applying acetone or a number of other solvents. The manner in which juvenile hormone analogue and the solvents exert their effect on diapause pupae remains unresolved, but the similarity of the response in temperate (Žďárek and Denlinger, 1975) and tropical flies suggests that the underlying mechanism is the same.

During diapause the metabolic rate drops to less than 15% of the lowest rate observed in non-diapause pupae at the same temperature. The low cost of maintenance thus permits diapause pupae to economically survive prolonged periods of dormancy. Since diapause in flesh flies coincides with the cool season of the year, the species is not confronted with the expensive type of dormancy (Janzen and Wilson, 1974) that is likely to occur in tropical organisms that diapause at high temperatures.

Mean QO_2 observed during diapause in the tropical species is quite similar to the QO_2 of temperate region flies (Denlinger *et al.*, 1972): 7.9 and 10.9 $\mu\text{l/g hr}$ for two African flies at 15° C compared with 8.5 $\mu\text{l/g hr}$ for the North American species *S. crassipalpis* at 18° C. For flies from both regions, however, QO_2 during diapause is not constant, as it is in diapause pupae of Lepidoptera (Schneiderman and Williams, 1953; Buck and Keister, 1955). Days of high metabolic activity (30–80 $\mu\text{l/g/hr}$) in the tropical flies are offset by days in which oxygen consumption is non-detectable. For temperate region flies, cycles of oxygen consumption are extremely regular, although cycle periodicity is distinctly greater during mid-diapause. In tropical flies, cycle periodicity is much more erratic and period length is not well correlated with pupal age. The basis for the sporadic QO_2 spikes is not yet clear, but we have proposed (Denlinger *et al.*, 1972) that metabolic end products may accumulate to a critical level and trigger a period of high metabolic activity that oxidizes the metabolic end products and concurrently generates sufficient high energy compounds to maintain the pupa until the next QO_2 spike.

In many respects, diapause observed in the tropical species is very similar to diapause of their temperate zone relatives: the developmental stage of diapause (young phaenoccephalic pupa) is identical, patterns of metabolic activity are similar and the neuroendocrine basis for the developmental arrest appears to be the same. The similarity suggests a common origin. Does this imply a tropical origin for the conspicuous dormancy in temperate zone flies or does diapause

capacity in the tropical species merely represent a relic from a temperate region evolutionary history?

The hypothesis proposed here is that diapause in flesh flies originated in the tropics and became the evolutionary basis for the long periods of dormancy observed in their temperate zone relatives. Taxonomic evidence (Rohdendorf, 1967) supports a tropical origin for flesh flies, and it is possible that primitive flesh flies radiating into temperate zones were, like their present counterparts in the tropics, already equipped with a mechanism for developmental arrest. Although the contribution of diapause to the life history pattern is not nearly as conspicuous among tropical fly populations, diapause does occur at a low level in the field (Denlinger, 1978). If the diapause were a relic not presently representing an adaptive advantage to the species, it is not likely that it would be retained so tenaciously. It can readily be eliminated by genetic selection as shown in *P. spilogaster*, and from laboratory rearing experiments it is apparent that removal of selective pressure for diapause, as in long-established laboratory colonies of *S. bullata* (Denlinger, 1972a), results in a decline of diapause expression. But, rather than being lost, the capacity for diapause appears to be actively maintained in each field population near the fringe of ecologically relevant temperatures. With a basic mechanism for developmental arrest already intact, flies migrating out of the tropics could supplement cold temperature signals with photoperiod cues to reliably forecast the long winter of the temperate zone. To extend the dormancy period nine months at 40° N (Denlinger, 1972b) required a major supply of stored energy. This could have been obtained readily by reallocating pupal energy reserves previously utilized by adults for egg maturation. Tropical species carry sufficient reserves through the pupal stage to enable adults to mature a major batch of eggs without first seeking a protein meal (Chadha and Denlinger, 1976). By contrast, their temperate region relatives either completely lack the capacity for autogenous development or, at best, have a limited capacity to mature eggs unless they, as adults, receive protein (Denlinger, 1971a; Baxter, Mjeni and Morrison, 1973; Pappas and Fraenkel, 1977). Thus, it seems possible that flies migrating into temperate zones extensively depleted their energy stores by investing in pupal diapause and became dependent upon an adult protein meal for reproduction.

Several colleagues contributed significantly to this research: Stephen Othieno provided able technical assistance, R. D. Ward supplied the colony of *S. ruficornis*, F. Zumpt and J. P. Reed identified the African flies, H. de Souza Lopes identified the species from Brazil, J. de Wilde and T. R. Odhiambo enthusiastically supported the research carried out at I.C.I.P.E., and J. de Wilde, P. Scheltes, J. Y. Bradfield, M. P. Murtaugh, G. P. Walker, and L. P. Lounibos commented on the manuscript. The research was supported in part by a postdoctoral fellowship from NIH-NIAID.

SUMMARY

1. Pupal diapause in flesh flies collected within 10° of the equator may be induced by low temperature but not by photoperiod. The threshold for diapause

appears to be near the fringe of ecologically relevant temperatures in each geographic area.

2. Cool temperature during the photophase elicits both a higher incidence of diapause and longer diapause than cool temperature coinciding with the scotophase.

3. Diapause persists for several months at a constant cool temperature, but four days at 25° C is sufficient to break diapause in *P. spilogaster*. The four-day exposure can be allocated either as a single block of time or as four one-day units spread over one month, suggesting that the fly pupa has an accurate mechanism for temperature summation.

4. Water content of the larval food exerts a minor effect on pupal diapause incidence: 10% addition of water elevated the response by 10%, and 10% decrease of water reduced the response by 18%.

5. Males and females enter diapause in approximately equal proportion.

6. When non-diapausing flies were selected for propagation, the level of diapause in *P. spilogaster* at 15° C dropped from 80 to 10% in ten generations.

7. Metabolic rate during diapause (7.9–10.9 $\mu\text{l O}_2/\text{g hr}$) is less than 15% of the lowest rate measured in non-diapause pupae. Infradian cycles of O_2 consumption similar to, but more variable than, the pattern in temperate zone flies are observed during diapause.

8. Adult development can be initiated readily with an injection of ecdysterone. A juvenile hormone analogue is ineffective by itself but greatly enhances the ecdysterone response. Development also can be triggered with topical application of several organic solvents.

9. Diapause in the tropical flies is proposed as the evolutionary basis for the long over-wintering diapause in flesh flies that have radiated into temperate zones.

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THE RELATIONSHIP OF SEASONAL CHANGES IN THE BIO-
CHEMICAL COMPONENTS TO THE REPRODUCTIVE
BEHAVIOR OF THE INTERTIDAL SPONGE,
HALICLONA PERMOLLIS

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Determination of seasonal changes in the levels of biochemical components of sponges sets a basis for studies on the metabolic requirements for their growth and reproduction and also establishes the nutritive quality of sponges for their predators. However, few studies have attempted to correlate environmental and metabolic events in sponges. Giese (1966) presented the average composition of marine invertebrates, including those of three species of sponges, *Haliclona permollis*, *Plocamia karykina*, and *Rhabdodermella nuttingi*. In a more recent study, Stone (1970) followed the annual fluctuations in biochemical and inorganic components in the intertidal sponge, *Hymeniacidon perleze*. The rates of accumulation of sponge biochemical components and their use for growth and reproduction have yet to be fully explored.

Sponges present unique procedural problems for determining component levels. The encrusting colonies vary considerably in size, and thus data cannot be based upon a "unit" animal. Varying water content does not allow use of wet weights. Data based on dry weight may incorporate an error as a result of remaining seawater salts and the presence of sand grains. Furthermore, percentage dry weight does not take into account the seasonal variation in the level of the inorganic component, the spicules, as demonstrated by Stone (1970). Finally, the application of percentage dry weight for any animal tends to mask the appearance of new levels of biochemical components, since the value of the component affects both the numerator and the denominator of the ratio.

This paper describes alternative procedures, based on skeletal weight and volume, for estimating the major biochemical components of sponges. The methods were applied to samples collected during the period August, 1970 to February, 1972, from an intertidal population of *Haliclona permollis* at Yaquina Head, Oregon. The sampled specimens were divided according to microhabitat and sex. The second year of biochemical data was correlated with the results of the study on reproduction and growth presented in a previous paper (Elvin, 1976a). To further investigate these correlations, observations were carried out on filtration rate, the effects of light on growth, and food utilization.

MATERIALS AND METHODS

A specimen of *H. permollis* was collected from each of ten sites of varying microhabitats in the rocky intertidal region at Yaquina Head, Oregon, on a

monthly basis. Three samples of each specimen were immediately divided into a portion preserved in Bouin's fixative for histological studies, a portion dried at 68° C for biochemical analyses, and a portion dried at 68° C, weighed, and then ashed at 580° C for determination of inorganic weight.

The component separation scheme employed was adopted from both Giese (1966) and Shibko *et al.* (1967). Approximately 50 to 100 mg of dried material was treated with 2:1 chloroform-methanol. The extracts were then dried under a nitrogen stream, and the lipid fractions spectrophotometrically determined by the dichromate oxidation method of Entenmann (1957) using palmitic acid as a standard. In a few cases, a second aliquot was redissolved in petroleum ether and the lipid components separated on a silica gel column following the method of Floch, Lees and Stanley (1956). Chlorophyll and carotenoid levels in April and May were determined using the optical density measurements of acetone extractions at three wavelengths as described by Strickland and Parsons (1968). The remaining tissue was extracted twice with cold 10% trichloroacetic acid (TCA), the supernatant brought to 60% ethanol, and sodium sulfate added as a coprecipitant (Van Handle, 1965). The resulting precipitate was considered to contain glycogen and was analyzed for glucose using the anthrone method (Seifter *et al.*, 1950).

The TCA insoluble material was redissolved with 1 N potassium hydroxide, incubated up to 20 hr at 37° C, neutralized with hydrochloric acid, and brought to a concentration of 2.5% TCA. The material remaining in solution was considered ribonucleic acid and quantitatively analyzed using the orcinol method for pentoses (Schneider, 1957). The remaining precipitate of protein and DNA was redissolved and the protein analyzed using the biuret procedure (Layne, 1955). DNA content was determined using the diphenylamine method of Schneider (1957). The remaining siliceous spicules were washed, dried at 70° C, and weighed. Microscopic examination did not indicate any appreciable degradation of the spicules even though they spent about 20 hr in an alkaline solution.

The biochemical data was based on both weight of inorganic material (spicules) and histological volume. The relationship between spicule weight of the samples and histological volume was determined by counting the number of spicules observed in a total of forty 400 power microscopic fields of 10 μ sections and obtaining a value for the number of spicules per mm³ (spicule density). The average spicule volume was determined from diameter and length measurements of 18 spicules per specimen. These sample sizes were sufficient to give a mean with 10% confidence intervals. Total spicule volume per mm³ of sponge was found by multiplying the number of spicules per cc of tissue times the average spicule volume, then the total spicule weight per mm³ was calculated using the value of 1.93 gm/cc for spicule density as given by Shore (1972). In this way the histological volume represented by a mg of spicules was determined, and the levels of biochemical components could be given in terms of weight per unit of sponge volume, henceforth known as component density, for any particular specimen. This method allowed quantitative correlation between biochemical levels and the reproductive and growth stages of the specimens.

Following a review of preliminary results, feeding rate and the effects of light on growth and feeding were investigated. During September, 1970, specimens were suspended in laboratory tanks subjected to a normal light regime

TABLE I

Maximum variation of assay procedures, and samples.

Component	Coefficient of Variation $\times 100$			
	Procedure	Individual	Sex	Population
Spicules/section*	—	7.0	—	9.0
Spicule volume	—	9.6	—	16.0
Histological volume	—	—	M	16.0
Protein/mg I	0.7	4.1	M + U**	14.0
			F	23.0
Protein/mm ³	—	—	M + U	19.0
			F	8.9
Lipid/mg I	1.6	2.3	M + U	14.0
			F	30.0
Lipid/mm ³	—	—	M + U	18.5
			F	24.0
Glycogen/mgI	12.6	20.6	M + U	60.0
			F	21.0
Organic/mg I	1.5	13.6	M + U	11.7
			F	25.2

* Section was a $40\times$ microscopic field of a 10μ section.

** Specimens in which sex could not be distinguished.

while other specimens were placed in the same system but in a tank wrapped in black plastic to eliminate light. The damp weights of the specimens were recorded for 29 days.

In order to determine filtration rates, sponges were collected attached to rocks, kept in the laboratory seawater system at the environmental temperature (9°C) and given a supply of the flagellate *Isochrysis galbana*. Filtration rates were determined by placing the sponge in 1 l of filtered seawater containing specimens of ^{14}C -labeled *Isochrysis* at the maximum concentration found in their natural environment (1800 cells/ml). Water samples were taken from a control tank without a sponge and from waters in which a sponge had been placed for 2 to 5 hr. Water samples were filtered with an 0.8μ millipore filter, air dried, and counted with a Gieger-Muller tube. The filtration rate was then estimated

$$(\ln C_0 - \ln C_t) / (\text{Volume})$$

by the formula: Rate = $\frac{(\ln C_0 - \ln C_t) / (\text{Volume})}{\text{Time}}$, where the initial and final con-

centrations of the flagellates, C_0 and C_t , were determined in units of counts per minute. The entire sponge was then scraped from the rock, dried, and weighed. Filtration rates were measured at times corresponding to different periods of the tidal cycle on the outer coast, in light and in darkness, and at the limits of the seawater temperatures, 9° and 15°C .

RESULTS

The variance for each biochemical component was established and divided into assay, individual, and population components as described by Holland and Gabbott (1971). The maximum coefficients of variance seen for each type of

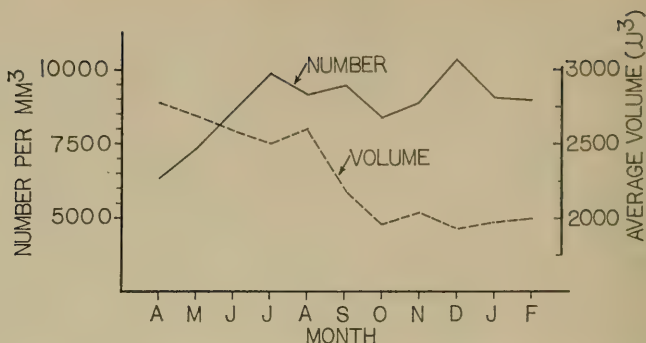


FIGURE 1. Characteristics of spicules in monthly histological samples. Solid line gives number of spicules in a mm^3 . Dashed line gives the volume of an average spicule.

measurement are given in Table I and can be used to determine the significance of the trends and differences in the data. In most cases, female sponges have higher variation than the group of males and indistinguishable specimens. Individual variation ($s/\bar{X}$) for proteins and lipids is less than 4%, while that for the organic/inorganic ratio and glycogen is larger.

The number of spicules per mm^3 of sponge was inversely proportional to the size of the spicules (Fig. 1). Individual spicule volume was greatest in spring, when spicule density was 7000 per mm^3 , and lowest in winter, when there were 11,000 spicules per mm^3 . As Figure 2 shows, the specific volume (mm^3 sponge/mg spicule) is highest during the fall (30) and lowest during the summer (20). The ratio of oxidizable organic substances to inorganic material (O/I) ranges from 1.2 in September to about 0.7 during April. During the months in which O/I was highest, no significant difference could be demonstrated between specimens from a microhabitat with a 3 hr daily air exposure period and those which had been continually submerged. The ratio based on spicule weight (O/S) is twice as high as the ratio of organic to inorganic components and differences between samples are more pronounced. The O/S ratio peaks approximately 1 month before that of the specific volume.

Beginning in February, the protein samples could be divided into two populations even though there is no apparent sign of sexual difference. The rest of the year female sponges with either eggs, embryos, or larvae had higher protein values than males. The amount of protein ranges from 300 (males, April) to 900 (females, October) $\mu\text{gm/mg}$ inorganic material (Fig. 3). An additional 5 to 8% was found associated with the spicule residue. Protein increases occurred during the periods of oogenesis and spermatogenesis but not during initial stages of embryogenesis. Females showed a dramatic decrease in protein content during the period of larval release from June to July.

In Figure 4 the data is presented in terms of component density and ranges from 10 to 30 $\mu\text{gm protein/mm}^3$. From this perspective, females again show increases from April to June during the period of oogenesis and embryogenesis and possibly a decrease from June to July. The increase during August was not seen in females as it was in males, although RNA levels continued to increase in both sexes at this time. Such data appear to contradict the large amounts of protein seen in the method based on inorganic weights. However, since protein level (P, S) and protein density (P/mm^3) are related by $(P/S) \div (\text{mm}^3/S) = (P/\text{mm}^3)$, and the histological volume (mm^3/S) is high at this time, the discrepancy disappears.

The amount of chloroform-methanol extractable lipid ranges from 44 (males, April) to 213 (females, September) $\mu\text{gm lipid/mg spicule}$ (Fig. 3). Increases in female levels were found from March to April associated with oogenesis and from May to September during the accumulation of mesenchymal amoebocytes. In the female portion of the sample there was a dramatic decrease in lipids during embryo formation and a smaller one from June to July, as was seen with protein during the period of larval release. Judged on the basis of specific density, there was a steady increase in $\mu\text{gm lipids/mm}^3$ during spring and summer (Fig. 4). As with protein, the large increases in lipid density were reduced

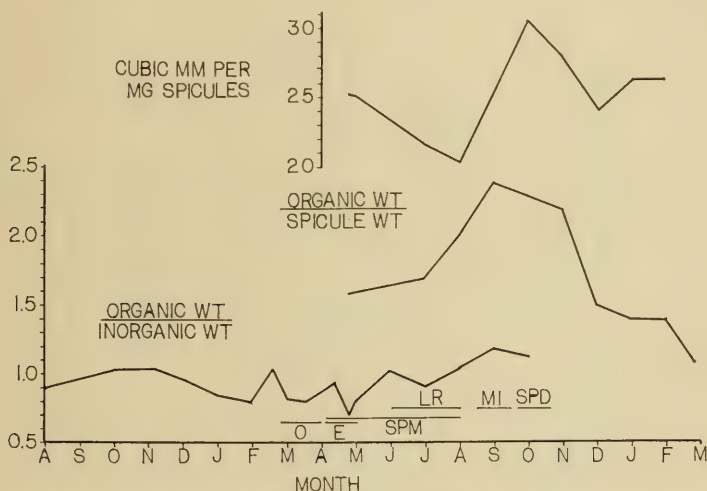


FIGURE 2. Ratios of organic to inorganic weights, organic to spicule weight, and specific volume of sponges for the period August, 1970 to February, 1972. Growth and reproductive periods indicated as O for oocyte production, E for embryo production, SPM for sperm production, LR for larval release, MI for peak of mesenchymal index, and SPD for peak of spreading period.

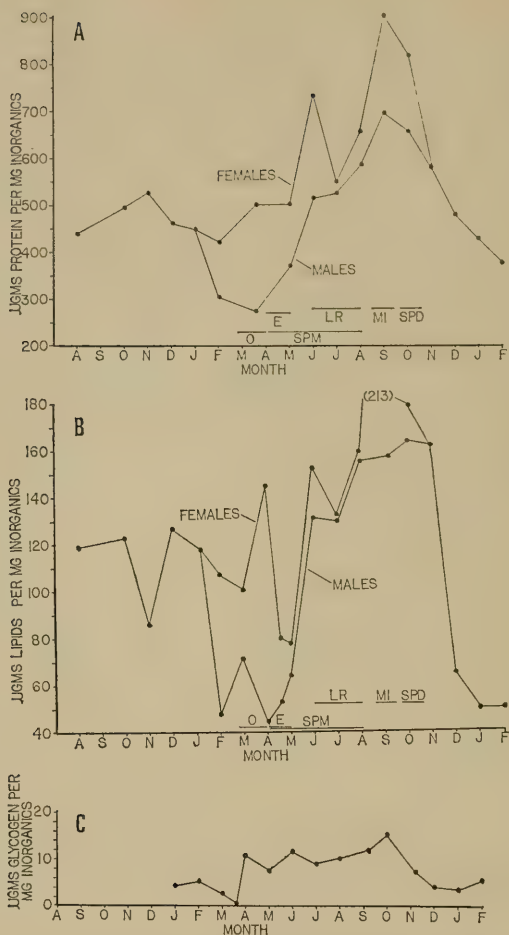


FIGURE 3. The amount of protein, lipid, and glycogen per mg of inorganic material for the period August, 1970 to February, 1972. Growth and reproductive period symbols as in Figure 2.

due to increases in histological volume during the fall. The surprising peak in November is statistically significant.

The lipid components of 10 specimens were separated by silica gel chromatography for the months August, October, November, and April corresponding to the times of highest lipid content. An average 44% of the lipids exist as polar or phospholipids. The sterol and free fatty acid component accounts for 20%. Monoglycerides comprise 21% of the total. The triglyceride fraction ranged from 1.7 $\mu\text{gm}/\text{mg}$ inorganics in a male collected in April to 10.4 $\mu\text{gm}/\text{mg}$ inorganics found in a female. These values are typical of the range of individual lipid fractions. Of the acetone extractable pigments, approximately 0.5 $\mu\text{gm}/\text{mg}$ inorganics were found to be chlorophylls and 1.0 $\mu\text{gm}/\text{mg}$ inorganics to be carotenoids.

Of the 50 to 80 μgm carbohydrates/ mg inorganics present, glycogen is found to range from 0 in the winter, to rise rapidly in April, and to be a maximum of 20 $\mu\text{gm}/\text{mg}$ inorganics in late fall. Based on volume, glycogen levels may reach 1

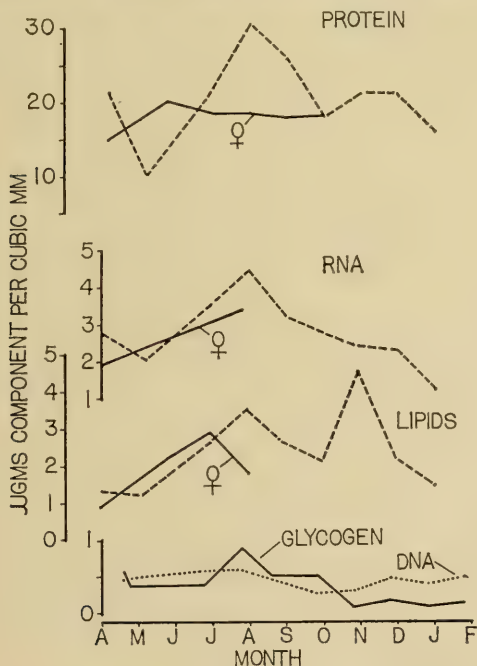


FIGURE 4. Weight of biochemical components per mm^3 of histological volume for the period April, 1971 to February, 1972.

$\mu\text{gm}/\text{mm}^3$. As with other components, the major period of decrease in this component occurs between October and November. The nonprotein components were found to be surprisingly high. Ribonucleic acid was present in amounts up to $70 \mu\text{gm}/\text{mg}$ spicules and DNA averaged $1 \mu\text{gm}/\text{mg}$ spicules.

A formula to estimate the protein and lipid required for growth and reproduction in terms of component density, C_{total} , was obtained by multiple regression. The regression constants, K_0 , K_1 , K_2 , and K_3 of the formula $C_{\text{total}} = K_0 + K_1 (\text{MI}) + K_2 (\text{OD}) + K_3 (\text{ED})$ were found to be 15.8, 4.6, -0.14 , and 0.43 , respectively for protein, and 1.2, 0.7, -0.02 , and 0.11 , respectively for lipid. Values for mesenchymal index, MI, oocyte density, OD, and embryo density, ED, are given in Elvin (1976a).

As Figure 5 shows, the sponges in the dark steadily declined in weight (3 to 4% loss per day) while there was little or no weight loss (0 to 1.7% per day) in the specimens living under the normal light regime. No significant difference was found between filtration rates of sponges in light with respect to those in darkness, and although rates were higher at 15°C than at 9°C , the range of values was also great. Filtration rates taken at the time of high tide in the intertidal were 5.5 times higher than those taken at periods of low water height. High tide values averaged 0.05 and ranged from 0.01 to $0.09 \text{ ml/minute per mg dry weight}$.

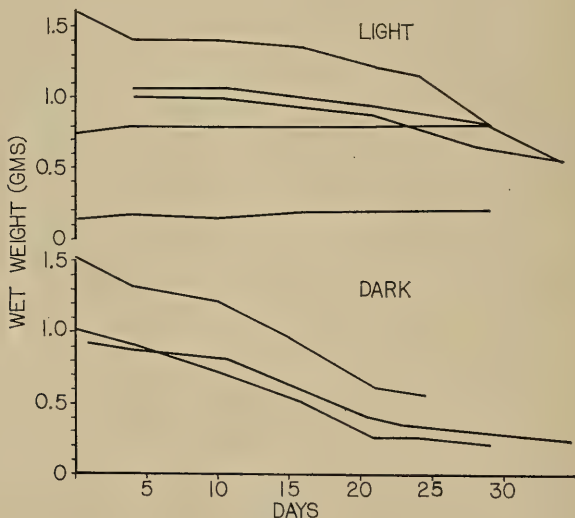


FIGURE 5. Change in total wet weight of sponges grown in normal light and in darkness for 30 days during the month of April.

DISCUSSION

Investigations of the relationships between environmental events and physiological processes require data on the rates of biochemical change. Calculation of these rates for the sponge, *Haliclona permollis*, based upon weight of skeletal structures or histological volume, allows correlation with the rates of growth and reproduction. Once the use of incorporated materials and energy has been determined, hypotheses concerning the life cycle strategy of these animals may be constructed.

Close consideration of the basis of calculation is very important for this animal, which lacks definite shape. Calculations based on dry weight, amounts of inorganics, or amount per animal lead to increased variability. For sponges the O/S ratio can be twice as high as the O/I ratio as a result of salts in the inorganic component. If salinity of the seawater is about 30‰, a cubic centimeter of wet sponge could contain up to 30 mg of salts or 27% of its estimated 80 mg dry weight. Since it is difficult to drain equivalent amounts of water from two sponges and since some of this salt is volatilized during drying (Paine, 1964), this source of error is both large and inconsistent. Thus, changes in biochemical levels based upon spicule weight should present a more accurate estimate.

There are still two sources of error in calculating biochemical components unique to this phylum. The first arises because spicule density can alter the apparent levels of the biochemical components. The shape and number of spicules in a sponge are influenced by silicon content, temperature, and food availability of the water (Elvin, 1971; Pé, 1973). Nearshore silicate values for the Oregon Coast have been shown to be minimal during spring and high during winter and periods of summer upwelling (Stefansson and Richards, 1963). Therefore, according to Pé (1973), small numbers of large spicules are produced under winter conditions of low nutrients and high silicon concentrations. The heavy spring skeleton is a result of spicule production in the previous winter, and the more numerous, smaller spicules develop during the summer and fall. Therefore, the accumulation of biochemical components in the summer and fall may be underestimated, since skeletal material is being produced at this time.

A second source of error is the change in biochemical level due to spreading or aggregation of amoebocytes rather than synthetic processes or utilization. Both of these problems may be corrected by dividing levels based on spicule weight by specific volume (mm^3/mg spicule) to give component density ($\mu\text{gm}/\text{mm}^3$). Treatment of the results in terms of component density demonstrates a slightly different picture than that presented by the values based upon weight. Using both methods, increase in size can be distinguished from lack of accumulation, and the influence of spicule production can be separated from biochemical data. In addition, data based upon volume allows rate calculations which directly correspond to the reproductive rate calculations previously reported (Elvin, 1976a). When either kind of data was converted into percentage dry weight, the amounts of the basic components, seasonal trends, and coefficients of variance were consistent with those given by Giese (1966).

When the biochemical values are separated according to the sex of the specimens, a number of correlations can be made with growth and reproductive processes.

Reproductive behavior for this population was described by Elvin (1976a) and is summarized at the base of the figures depicting protein and lipid levels (Fig. 3). Oogenesis begins in late February to early March, embryos and sperm are first seen in April, and larvae are released from sponges during the summer. During the fall, a considerable increase in the density of amoebocytes can be noted and these cells then spread outwards during the late fall, increasing the size of the sponge. This period of spreading is followed by a decline or regression of cells during the winter.

Based on protein and lipid values, sexual differentiation can be detected before gametes are recognizable. In February the sampled populations can be divided into two groups. The values for specimens with gametes found in early March imply that the February specimens with the higher amounts of protein and lipid represent females prior to gametogenesis. A significant drop in protein and lipid from January to February is seen and an increase in the component values does not occur until spermatogenesis is taking place (April–August). This latter increase merges with the fall somatic growth.

In contrast to males, the level of protein and lipid in females begins a rise directly correlated to oocyte production during February to April. This material is probably going into nurse cell production rather than directly into oocytes. The period of embryo formation, April to May, is particularly interesting since protein shows no increase and lipids decrease to half their value.

On the other hand, lipid density (Fig. 4) shows a continual rise during this period. Part of the explanation for this contradiction is found by looking at the rate of spicule formation. Data in Figure 1 and Figure 2 give evidence for initiation of spicule production in May. This change in the inorganic component would decrease the apparent levels of biochemical constituents and specific volume, and thus mask changes in these parameters. In this case, a decrease in specific volume will increase lipid density.

A second interesting possibility might be found in the phenomenon of sponge embryogenesis. Fell (1969) noted that as embryogenesis progresses, the size of the nurse cells which have been incorporated into the oocyte decreases. Lipid could be used as an energy source for this process. In the period following embryogenesis the amounts of both protein and lipid increase until the time of larval release. About 25% and 15% of the protein and lipids, respectively, are lost at this time.

Growth occurs mainly during the fall (Elvin, 1976a) and involves far greater accumulation of materials than reproduction. As the mesenchymal index rises from August to September, the proteins and lipids increase to nearly double their previous amount. Sponges during the fall of 1971 had almost twice the protein and lipid per mg spicules as those of 1970. Even though reproduction has been completed, the sexes can still be distinguished through the presence of residual gametes. It is found that females still accumulate more materials per mg spicule than males. This difference may be an environmental effect rather than a physiological one, since a higher percentage of males are found in more exposed microhabitats (Elvin, 1976a). Sponges in these regions reach higher temperatures, and undergo greater dessication, and such conditions may cause greater stress. The large fall protein peak for females is not seen in the data based on volume.

TABLE II

Estimates of protein requirements for three processes ($\mu\text{gm}/\text{mm}^3/\text{day}$).

Process	Period	
	April-May	October-November
Somatic	0.22 (54%)	0.10 (19%)
Reproductive	0.06 (15%)	0 (0%)
Spreading	0.13 (32%)	0.44 (81%)
Total	0.41 (100%)	0.54 (100%)

The high values per mg spicule and the low values per mm^3 of histological volume indicate increased components and volume without increases in spicule growth. Males seem to have increases in spicules in addition to increases in component density. The possibility exists that there are physiological differences in sexes in addition to those involved in gamete and embryo production. The high peak in November is real and remains unexplained. Possibly, it is related to increase in particulate matter captured from the water. Glycogen is present in less than one-tenth of the amount of protein and lipids. It accumulates during spring and is used in the late fall before the drop in lipid.

The decline in the components based on inorganic weights occurs with spreading of the animal over the substrate. As the amoebocytes move from the old skeleton into new areas, the amount of biochemical components relative to the spicule weight decreases. The same picture is seen in the data based on volume. Further studies of this nature would require 30 to 40 specimens of each sex to attain a mean with 10% confidence intervals. Since such collections would be time consuming and disrupt existing populations, alternative methods must be sought for further understanding of the biochemical events.

The relatively large values of the intercepts and standard errors in the component regression equations reflect the inaccuracy of the subjective mesenchymal index, the variation in embryo size, and the small sample size. The regression coefficients indicate that oogenesis involves little accumulation of nutrients and may even result in a slight loss of protein. Embryo formation involves an accumulation of $0.43 \mu\text{gm}$ protein/embryo. Therefore, at the peak of embryo production during July, when the average embryo density is $3.5 \text{ embryos}/\text{mm}^3$ and the maximum $20 \text{ embryos}/\text{mm}^3$ (Elvin, 1976a), the ratios of "reproductive" protein to total protein are 1.5/18.6 and 8.4/27.9 or 8% and 30%, respectively.

During the spring of 1971, embryo production averaged $0.14 \text{ embryos}/\text{mm}^3$ per day, which requires $0.06 \mu\text{gm}$ protein/ mm^3 per day. In the spring of 1973, this requirement reached $0.17 \mu\text{gm}$ protein/ mm^3 per day. During this period the average total protein increased at a rate of $0.22 \mu\text{gm}$ protein/ mm^3 per day and the rate of increase in size (spreading) was $0.65 \text{ mm}^2/\text{cm}^2$ per day. If one assumes that spreading in two dimensions is equivalent in amount to increase in volume, then the protein lost from any particular volume due to spreading is about $0.65\% \times 20 \mu\text{gm}$ protein/ mm^3 or $0.13 \mu\text{gm}/\text{mm}^3$ per day. During the fall, the spreading component reaches 2.2% or $0.44 \mu\text{gm}$ protein/ mm^3 per day. Table II summarizes the partitioning of the protein demands. Although material allocated for embryo

production has been estimated at 2% based on relative volumes (Reiswig, 1973; Elvin, 1976a), the estimates in terms of rates of protein requirement seem to be about 10 times higher.

It is reasonable to assume that environmental temperature and nutritive availability would influence rates of protein and lipid accumulation as well as internal physiological state. The thermal regime and seasonal variation in the available oxidizable particulate concentration of coastal seawater for this population have been presented elsewhere (Elvin, 1976a). Although thermal events in the environment may play a role in initiating reproductive processes, there is little indication that it is directly related to accumulation of the major biochemical components. There is a better correlation between the increased temperatures in late summer and fall with the somatic growth. The largest amounts of particulate material occur during April and May with slightly lesser quantities during fall.

The filtration rates correspond to those calculated by Frost (1976). A rough calculation using the value for a filtration rate of 0.05 ml/minute per mg dry weight, an average specific volume of 25 mm³/mg of spicules, and a concentration of particulates in seawater of 1 mg/liter (Elvin, 1976a) results in an estimated intake of 3 μ gm/day per mm³ of sponge, if one assumes 12 hr of submergence. This estimated food intake is one order of magnitude greater than the calculated requirements. Since most of the biochemical accumulation occurs during the fall, when particulates are not very high, either food consisting of particles 1 μ or larger is not limiting or it is not the predominant food source. Reiswig (1975) has demonstrated that bacterial and colloidal particulates are more important for this species.

No significant difference could be found between biochemical levels of those sponges subject to four hours of air exposure each day and those continuously submerged. The observations suggesting the presence of a tidal feeding rhythm would reduce the difference between food intake of sponge living in high and low levels of the intertidal zone.

Since weight decreases occurred more rapidly in sponges held in the dark, it is possible that this population may have its growth and distribution and possibly its reproduction influenced by the quality and quantity of light. Increased light may give this animal some nutritional advantage which would compensate for loss in feeding time during exposure. The presence of chlorophyll could be due to either phytoplankton trapped in the sponge canals, phytoplankton trapped on the surface, or possibly to algae in symbiotic relationship, as has been described by Sará and Vacelet (1973). The decreased body weight in those specimens kept in the dark might be related to this presence of chlorophyll. However, Rasmont (1970) reported an influence of light on growth of freshwater sponges which did not contain photosynthetic symbionts.

The life cycle strategy employed by this species located in the rugged environment of the intertidal zone appears to be one of modest reproductive effort and considerable investment in somatic growth prior to the harsh winter. Although this species does have gemmules, they were found infrequently and hence are probably not used for overwintering in this population. If this is the case, the female sponges with the greatest biomass production would have a better chance of surviving until the next spring. As pointed out by Elvin (1976a), it is not

known whether these second year females become males, females, or lack sexual expression.

Elvin (1976a) has shown a temporal separation between growth and reproduction. The exciting possibility of an inverse relationship between the biochemical components allocated to sperm and embryo production and those used for somatic growth should be investigated further. Do individuals using small amounts of protein on few embryos in the spring show particularly large somatic growth in the fall?

In comparison with other sponges in the intertidal region, *H. permollis* appears to be a good source of protein for its nudibranch predator, *Diaulula sandiegensis* (Elvin, 1976b). The levels of inorganic and biochemical components are similar to those found for the sponge *Halichondria panicea*, which is consumed by the nudibranch *Archidoris montereyensis* as described by Carefoot (1967). Randall and Hartman (1968) found that sponge-eating fishes also tend to choose those species with low spicule components. Sponges during the late summer have the greatest nutritive content per volume, and major nudibranch growth might be expected during this period. It is possible that the larger females with greater protein content are a more attractive food source for their nudibranch predators and would be selected as food over males. During July, 1971 a particularly large number of *D. sandiegensis* was noted in the collecting area and presumably these individuals would grow quite rapidly during the next 3 months.

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SUMMARY

1. Biochemical requirements for growth and reproduction in the intertidal sponge, *Haliclona permollis*, were estimated by following the total amounts of protein, lipid, glycogen, RNA, and DNA. The ratio of total organic weight to spicule weight and the histological volume per unit spicule weight were also followed seasonally.

2. Protein and lipid accumulation in females began in February before oocytes were observed. Lipids were consumed during embryogenesis. Following the reproductive period, females continued to have greater accumulation of protein and lipids than males.

3. Daily protein requirements of a spring female were $0.22 \mu\text{gm}/\text{mm}^3/\text{day}$ for somatic growth, $0.06 \mu\text{gm}/\text{mm}^3/\text{day}$ for embryogenesis, and $0.13 \mu\text{gm}/\text{mm}^3/\text{day}$ for spreading. The requirement for spreading increases to $0.44 \mu\text{gm}/\text{mm}^3/\text{day}$ during the fall growth period.

4. The proportion of protein relegated to reproductive functions averages 8% of the total and can rise to 30% in females.

5. The life cycle strategy of this population appears to place most of the synthetic and accumulative efforts into fall somatic growth and spreading in order to allow increased survival over the winter and subsequent reproduction in the spring.

6. The sponge appears to be a particularly good source of protein for its principle predator, *Diaulula sandiegenesis*.

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REPRODUCTION AND DEVELOPMENT OF *HALICHONDRIA* SP. IN THE MYSTIC ESTUARY, CONNECTICUT¹

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Although sponges belonging to the genus, *Halichondria*, are often abundant in the coastal waters of temperate regions, little is known of their biology, including reproduction. Meeuwis (1941) made a detailed cytological study of embryonic and larval development in *Halichondria coalita* but did not examine gametogenesis. Bergquist, Sinclair and Hogg (1970) reported what they believed to be the asexual production of larvae, in addition to sexual reproduction, in *Halichondria panicea* and *Halichondria moorei*. However, it now appears that their interpretation is incorrect and that larvae are produced exclusively by a sexual process (Bergquist, personal communication, but see Evans, 1977). Bergquist and Sinclair (1968) described the behavior of the larva of *Halichondria moorei*, and Bergquist and Green (1977) and Evans (1977) examined the ultrastructural features of the larva of this species and its metamorphosis into a small sponge. The reproductive periods of *Halichondria bozverbanki* at New Haven, Connecticut (Hartman, 1958) and of *Halichondria panicea* at Auckland, New Zealand (Bergquist and Sinclair, 1973) have also been determined. While these studies provide a general picture of reproduction within the genus, *Halichondria*, there have been no detailed studies of the complete life histories of particular species. In fact, comprehensive studies on the reproductive biology of any sponge, fresh-water or marine, are few (See Fell, 1974a, 1976b for reviews).

The present study was undertaken as a first step in the analysis of reproduction in *Halichondria* sp. and includes information on sexual differentiation, gametogenesis, embryonic development, larval settlement, and reproductive period. Preliminary data on the reproduction of this species was presented earlier (Fell, 1974a) under the designation, *Halichondria bozverbanki*. However, subsequent examination of the free-swimming larva indicates that the sponge is a different species or subspecies.

During much of the summer and fall, the population of *Halichondria* sp. in the Mystic Estuary consists of two types of specimens: those which survived the preceding winter in a dormant condition and became active during the spring, called "postdormant specimens," and those which developed from larvae produced during the current year, called "postlarval specimens." The postdormant specimens are found resting on the bottom, while most of the postlarval specimens are attached to eelgrass. Since the eelgrass undergoes degeneration during the winter and regrowth during the spring, all sponges present on the standing eelgrass in

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TABLE I

Sexual differentiation in postdormant specimens of Halichondria sp. in the Mystic Estuary, Connecticut.

Year	Month	Number of specimens			
		Males	Females	Hermaphrodites	Total
1970	May	5	12	4	28
	June	17	10	0	28
	July	3	4	0	19
		25	26	4	75
1975	May	0	1	14	15

summer are clearly derived from larvae settling during the current year. However, some larvae may settle on the bottom and some postlarval specimens may fall to the bottom following fragmentation of the eelgrass. Consequently, it is not always possible to distinguish postlarval specimens, which are resting on the bottom, from postdormant sponges. The present study focuses attention on postdormant specimens but provides some preliminary information on the reproduction of young postlarval specimens.

MATERIALS AND METHODS

Specimens of *Halichondria* sp., which had overwintered in a dormant form (postdormant specimens), were collected from the lower part of the Mystic Estuary, Connecticut at regular intervals during the spring and summer of 1970 and additional collections were made during 1969, 1971, and 1975. Usually a minimum of six specimens were collected on each date. In most cases the specimens were fixed in seawater-Bouin's solution immediately following their collection. The fixed specimens were cut into pieces and examined under a dissecting microscope for the presence of large oocytes and embryos, and then small samples of the specimens were prepared for histological study. The samples were embedded in paraffin and sectioned serially at 10 μ , and the mounted deparaffinized sections were stained with hematoxylin and eosin. In all more than 150 specimens, collected during the period extending from April through September, were examined histologically. A study of specimens collected during the remainder of the year will be reported later as part of a consideration of winter dormancy.

During the summer of 1977, postlarval specimens encrusting the blades of the new crop of eelgrass were collected at regular intervals and examined under a dissecting microscope to determine whether embryos were present. No histological study was made of these sponges.

The plankton of the shallow lower estuary was regularly sampled for the presence of sponge larvae using a small, 20-mesh plankton net towed by boat. In addition, intact specimens of *Halichondria* were collected at regular intervals and brought back to the laboratory, where they were kept in freshly collected sea water for 12 to 24 hr to determine if they would release larvae. This procedure worked

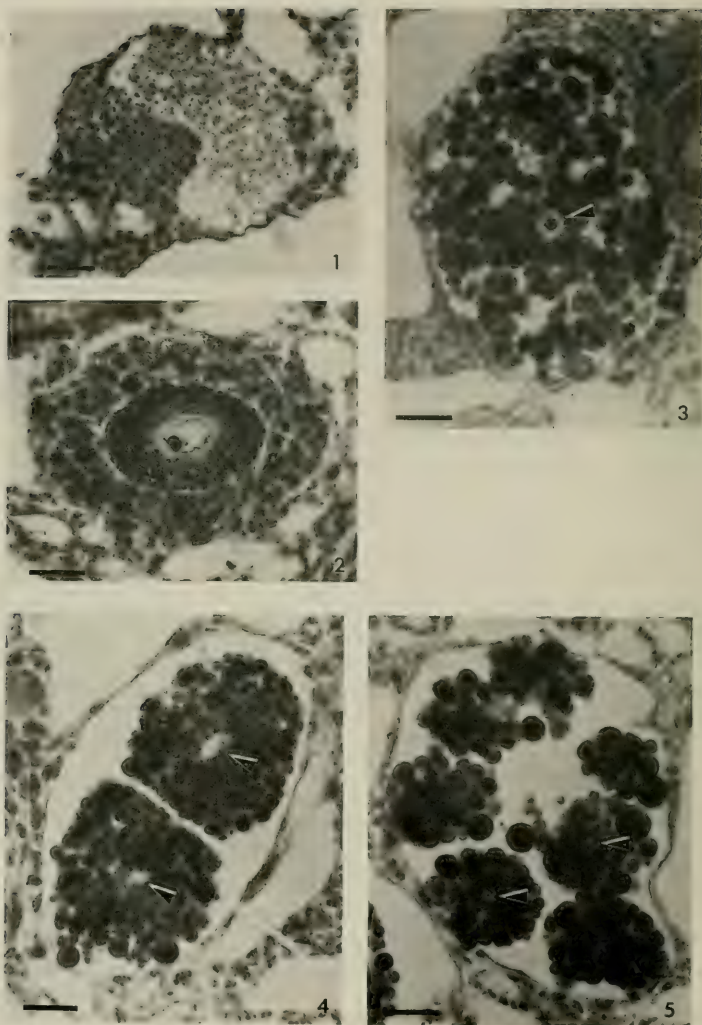


FIGURE 1. A spermatic cyst with cells in different stages of differentiation; scale bar is 25 μ .

well with small postdormant and with postlarval specimens. However, since large postdormant specimens collected during mid-summer frequently did not remain active and healthy throughout the period of observation, only limited information was obtained by this procedure.

Larval settlement was studied during 1971 by placing shells of the hard clam, *Mercenaria mercenaria*, in the Mystic Estuary at regular intervals. Freshly shucked shells were thoroughly cleaned in fresh water and completely dried. Then a small hole was drilled near the center of each shell, and the shells were strung in groups of five on nylon cord with the concave (inner) surface down and tygon tubing spacers (1.5 inches long) between successive shells. Ten shells were set out below the level of extreme low tides at the beginning of each three-week exposure period from 1 May to 16 August. Following removal from the estuary, the shells were brought back to the laboratory and immediately examined under a dissecting microscope for the presence of sponges. The identification of each small sponge was checked by preparing a wet mount of it and examining the preparation at a magnification of 430 $\times$ to observe spicule characteristics.

RESULTS

Gametogenesis and embryonic development

Information on sexual differentiation in *Halichondria* sp. suggests that this sponge may exhibit an incomplete gonochorism (see table I). During 1970, the population appeared to consist predominantly of distinct males and females which occurred in approximately equal numbers. However, there were a few contemporaneous hermaphrodites. By contrast, during 1975, essentially all of the reproductive specimens examined were hermaphroditic. In these specimens apparently normal male and female reproductive elements were intermingled, and some specimens contained large spermatogenic cysts in various stages of differentiation, oocytes, embryos and flagellated larvae. Furthermore, of 26 specimens examined during July and August of 1971, two were hermaphrodites, nine contained only small oocytes and none possessed spermatogenic cysts. While these data suggest that some specimens may form only one type of gamete and other specimens may form both, the occurrence of successive hermaphroditism cannot be ruled out. Clearly, more information on sexual differentiation in this species would be of considerable interest.

Spermatogenesis occurs within spermatogenic cysts which are enclosed by a simple squamous epithelium. The cysts, which may range in size up to more than 100 μ in diameter (Fig. 1), are scattered throughout the endosome. Usually, cysts in all stages of development are present in the same specimen, and more than one stage of spermatogenesis may be present in a single cyst. The mature sperm are small, the heads being spheres with an average diameter of about 1 μ . Spermatozoa appear to be oriented at random within the cysts.

FIGURE 2. An early vitellogenic oocyte containing small yolk granules and surrounded by a mass of nurse cells; scale bar is 25 μ .

FIGURE 3. A fully developed egg (or possibly zygote) containing large yolk spheres. Note the vesicular nucleus with prominent nucleolus (arrow) in the center of the egg and the thin follicular epithelium surrounding the egg; scale bar is 25 μ .

FIGURES 4 and 5. Early cleavage stages. Nuclei are indicated by arrows; scale bars are 25 μ .

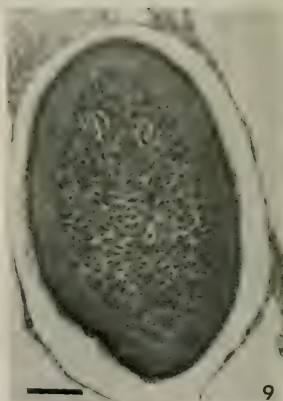
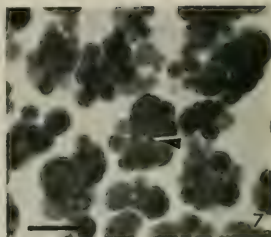
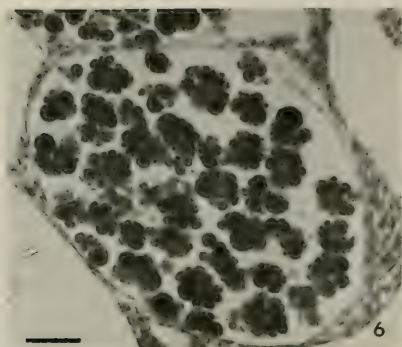


FIGURE 6. A later cleavage stage which still contains large yolk spheres within the cells; scale bar is $42\ \mu$.

FIGURE 7. An enlarged portion of the same embryo shown in Figure 6. A nucleus is indicated by an arrow; scale bar is $25\ \mu$.

FIGURE 8. A developing larva in which the cells have segregated into a peripheral region and a central cellular mass. A large persisting yolk sphere is indicated by an arrow; scale bar is $42\ \mu$.

Oocytes and embryos usually develop individually within the endosome, but in some specimens clusters of 10 or more previtellogenic oocytes occur. In many cases all stages of oogenesis and embryonic development are intermingled in the same specimen. The initial growth of the oocytes is accomplished without the formation of yolk granules. Then, as the oocytes begin their major period of growth (at ca 35 μ diameter), they become surrounded by a mass of nurse cells and small yolk granules accumulate in the cytoplasm, except for the region immediately surrounding the nucleus (Fig. 2). Later, large yolk spheres, ranging in size up to about 17 μ , develop in the oocyte cytoplasm (Fig. 3). Many of the larger yolk spheres appear to consist of smaller granules and presumably form as a result of the fusion of the latter. The oocytes possess a large nucleus containing a prominent nucleolus.

Stages of meiosis and fertilization have not been observed in this material; however, the presence of specimens containing large numbers of sperm suggests that the form of reproduction described here is sexual.

A typical cleavage sequence is obvious in this species; embryos with from two to many blastomeres are clearly recognizable (Figs. 4-7). There is little breakdown of yolk spheres during early cleavage. However, as cleavage proceeds the yolk is gradually utilized; and by the end of cleavage, the embryo consists of a large number of small cells lacking distinct yolk spheres.

When the breakdown of yolk spheres is essentially complete, the embryo becomes divided into two parts: a central cellular mass and a peripheral region consisting of several cellular layers (Fig. 8). This division marks the beginning of larval development. The outer cells of the peripheral region align and form what appears to be a simple columnar, flagellated epithelium, while the inner ones form a more or less distinct subepithelial tissue (Fig. 9). In the definitive parenchymula larva, the flagella covering most of the surface are about 12 μ long but those at the posterior pole are about 36 μ in length. The central cellular mass forms a dense mesenchyme in which spicules develop. The larval spicules are thin oxeas about 130 μ long. They are oriented along the longitudinal axis of the larva and are concentrated toward the posterior pole.

The free-swimming larvae have a uniform bright yellow color. They typically exhibit a pear-like form, but some of them may possess the shape of a cylinder with rounded ends. Although the larvae vary somewhat in size, their dimensions are about 275 $\times$ 150 μ .

During some years (1969, 1970, 1974), the eelgrass, *Zostera marina*, of the lower Mystic Estuary became covered with large numbers of small postlarval specimens of *Halichondria*. As many as 50 sponges could be found attached to a length of eelgrass leaf 8 cm long. Many of the sponges were separated from one another by at least a small distance (a millimeter or more), but others were in close contact with each other or had apparently fused (Fig. 10). Although the evidence is circumstantial, it appears that postlarval fusion may be common in this species under favorable conditions. Also, many small postlarval specimens were observed

FIGURE 9. A fully developed parenchymula larva. Note the outer flagellated epithelium, the subepithelial tissue and the central region of dense mesenchyme; scale bar is 42 μ .

FIGURE 10. A sponge which has apparently resulted from the fusion of three postlarval specimens; scale bar is 6 mm.

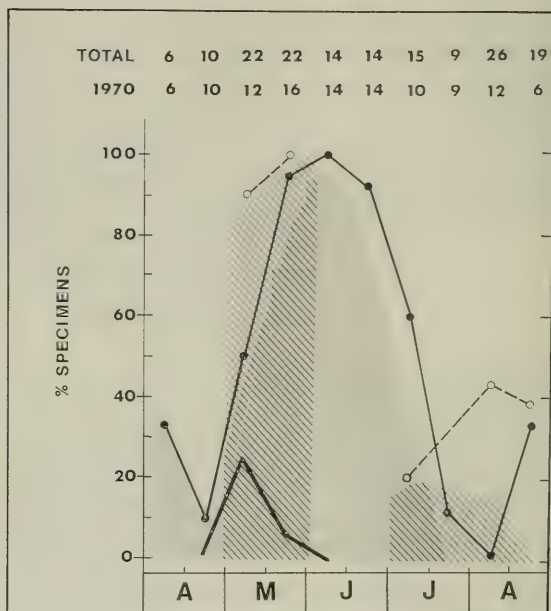


FIGURE 11. Reproductive activity of postdormant specimens of *Halichondria* sp. in the Mystic Estuary, Connecticut. Solid circles joined by heavy lines represent the percentages of specimens with some reproductive elements at various times during the spring and summer of 1970, and the gray region shows the percentages of specimens containing large numbers of reproductive elements during the same period. The open circles joined by broken lines indicate the percentages of specimens containing reproductive elements and the cross-hatched regions represent the percentages of specimens possessing large numbers of reproductive elements during other years. The very heavy solid lines show the percentages of hermaphroditic specimens during 1970. The number of specimens examined during each period is indicated at the top of the figure.

attached to blades of eelgrass floating freely in the water. The movement of sponges on such substrates would appear to provide an additional dispersal mechanism following dispersal and settlement of the free-swimming larva.

Reproductive period

During early April, many of the specimens of *Halichondria* sp. in the Mystic Estuary are still dormant (Fell, in preparation), but very soon after the sponges emerge from the dormant state, they commence reproductive activity. In April

TABLE II

Occurrence of various developmental stages in female postdormant specimens (including hermaphrodites) of Halichondria sp. in the Mystic Estuary during 1970. (Large oocytes are defined as those containing yolk granules; embryos are defined as cleavage stages and developing larvae.)

Month	Number of female specimens	Percentage females containing:			
		Small oocytes	Large oocytes	Embryos	Flagellated larvae
April	3	100	0	0	0
May	16	94	63	44	13
June	10	100	90	90	70
July	4	100	0	50	50
August	0	—	—	—	—

of 1970, only few specimens contained reproductive elements and all of these sponges possessed only small previtellogenic oocytes. During May and June of that year, the number of specimens containing reproductive elements increased (Fig. 11), as did the percentage of female specimens containing later developmental stages (Table II). Then, during July, reproductive activity declined, and by August few reproductive specimens were observed. In September, 10 of 12 sponges examined were reproductive; however, they contained only variable quantities of spermatid cysts or a few small oocytes.

The data available for other years suggest that the situation in 1970 was fairly typical. However, in May 1975, reproductive activity was apparently somewhat advanced compared to that during 1970. All of the specimens examined (15) contained large numbers of reproductive elements; 93% possessed embryos and 40% contained flagellated larvae.

A study of postlarval specimens during the summer of 1977 showed that some of them contained embryos (actually large oocytes, embryos and/or larvae) from early July until at least mid-October (Fig. 12c). Larvae were released in the laboratory by small postdormant specimens during late May and June and by postlarval specimens during August and September; and larvae were observed in plankton samples collected during a major portion of the combined periods (Fig. 12b).

During a number of years (1969, 1970, 1973, 1974), small postlarval specimens were observed on the new crop of eelgrass by mid- to late June. Such specimens may be extremely abundant beginning at this time. However, in 1971 larval settlement occurred on clam shells suspended in the estuary primarily during the period extending from late July into September (Fig. 12a). That this settlement was primarily due to larvae produced from postlarval specimens is suggested by the fact that, of 32 larger specimens collected during July, August, and September of that year, only two contained embryos and/or larvae. The occurrence of little detectable larval settlement earlier in the summer can apparently be explained by the scarcity of postdormant specimens during 1971. The low level of larval settlement on the clam shells in June and July was correlated with relatively little larval settlement on the eelgrass during this period.

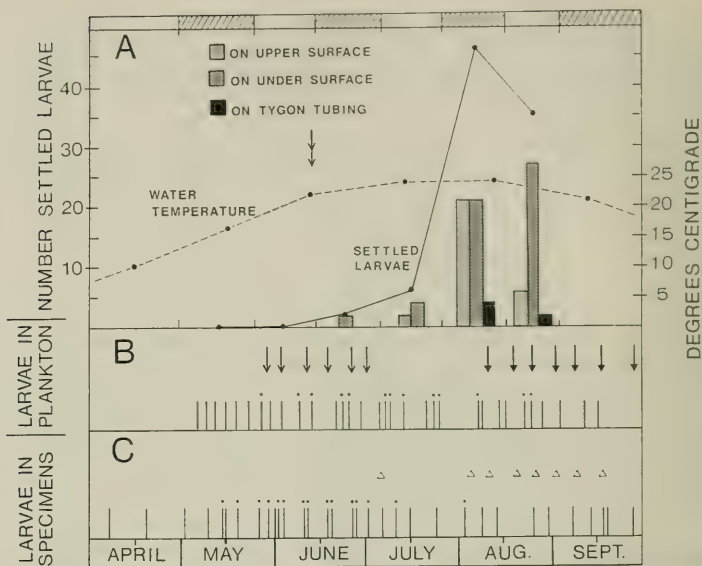


FIGURE 12. Summary of sexual reproduction by postdormant and postlarval specimens of *Halichondria* sp. in the Mystic Estuary, Connecticut. Surface water temperature and larval settlement are shown in A: right, monthly means in water temperature from data collected over a period of several years; left, the number of larvae settled on 10 *Mercenaria* shells per 3-week period. The shells were strung with the concave surface down and tygon tubing spacers between them. The pattern of settlement on available surfaces is indicated. The double arrow marks the earliest time that large numbers of small postlarvae were observed on eelgrass in the same area. The occurrence of larvae in plankton samples and the release of larvae by freshly collected specimens in the laboratory are shown in B: vertical lines show when plankton tows were made and dots indicate when larvae were found. Arrows with V-shaped heads denote release of larvae by postdormant specimens and arrows with solid heads indicate release of larvae by postlarval specimens. The occurrence of flagellated larva in postdormant specimens collected over several years and of embryos (actually large oocytes, embryos and/or larvae) in postlarval specimens during 1977 are shown in C: vertical lines indicate when postdormant specimens were collected; when dotted, flagellated larvae were observed in histological sections of the sponges. Open triangles show when embryos were found in postlarval specimens.

Nearly twice as many larvae settled on the under (inner) surface as on the upper surface (54 compared to 29) of the shells suspended in the Mystic Estuary (Fig. 12a). At the present time, the basis for this apparent preference is undetermined.

Taxonomic status

The sponge considered in this report resembles *Halichondria bowserbanki* (Hartman, 1958) in many respects, including general form, color, spiculation and winter

dormancy (Fell, 1976b). However, the free-swimming larva differs from that of *Halichondria bozzerbanki* in its pattern of flagellation (see Discussion). In this respect the larva resembles that of *Halichondria coalita* (Topsent, 1911), which, in spite of this difference, is considered by some authors to be synonymous with *Halichondria bozzerbanki* (Hartman, 1958). The situation is complicated by the fact that embryonic development of *Halichondria coalita* (Meewis, 1941) differs substantially from the process described here for the sponge in question (see Discussion). Because of these considerations, it seems wiser to refer to the sponge as *Halichondria* sp. than to assign it to either *Halichondria bozzerbanki* or *Halichondria coalita*.

Clarification of the taxonomic status of this sponge must await further studies of various members of the genus *Halichondria*. Developmental studies will be especially important.

DISCUSSION

Halichondria sp. appears to exhibit an incomplete gonochorism (or possibly successive hermaphroditism), with some specimens possessing only sperm or oocytes (and/or embryos) and other specimens simultaneously containing both male and female reproductive elements. During some years separate sexes appear to predominate, while during other years hermaphroditic specimens seem to constitute the bulk of the population. In many of the hermaphrodites, large numbers of spermatocysts, oocytes, and embryos are intermingled. The occasional occurrence of hermaphroditic specimens in populations of sponges largely composed of unisexual specimens has been reported for *Hymeniacidon* (*Stylotella*) *heliophila* (Fincher, 1940), *Haliclona ccbasis* (Fell, 1970), *Haliclona permollis* (Elvin, 1976) and a number of other species (see Sarà, 1974 for a review). However, the high degree of variability in the pattern of sexual differentiation found in *Halichondria* sp. has not been previously described. Clearly, it is necessary to study a sponge population over an extended period of time, using a variety of approaches, before definite conclusions about sexuality can be drawn (see Fell, 1976b).

There are conflicting reports in the literature concerning the sexual condition of certain species. For example, according to Leveaux (1941) *Spongilla lacustris* is a contemporaneous hermaphrodite, while Gilbert and Simpson (1976) have shown that *Spongilla lacustris* from a different region is a successive hermaphrodite in which sex reversal may occur from year to year following winter dormancy in the form of gemmules. In the latter population, no specimens containing both sperm and oocytes (and/or embryos) have been found. It is unclear whether such differences between populations reflect genetic diversity within the same species (or possibly different, closely related, species) or an inherent lability in the regulatory mechanisms which may be influenced by environmental factors (Elvin, 1976; Gilbert and Simpson, 1976). Van de Vyver and Willenz (1975) imply that sex reversal similar to that of *Spongilla lacustris* may also occur in *Ephydatia fluviatilis* (strain B).

Oogenesis in *Halichondria* sp. is similar to that of *Hymeniacidon* (*Stylotella*) *heliophila* (Fincher, 1940), *Hymeniacidon caruncula* (Diaz, 1973), *Ephydatia fluviatilis* (Leveaux, 1941, 1942; Van de Vyver and Willenz, 1975), and *Spongilla*

lacustris (Leveaux, 1941, 1942; Simpson and Gilbert, 1973; Gilbert, 1974). During their development, the oocytes become filled with yolk spheres ranging upward in size to about 15 to 20 μ . This situation differs from that found in *Halichondria coalita*, in which the eggs possess a homogeneous cytoplasm containing only small yolk granules (Meewis, 1941).

Embryonic and larval development in *Halichondria* sp. closely resemble these processes in *Halichondria panicea* (figured by Fell, 1974a). Cleavage is followed by the formation of a very compact parenchymula larva which is completely covered by flagella and contains a central bundle of spicules. However, unlike the larva of *Halichondria panicea* (Topsent, 1911), the larva of *Halichondria* sp. possesses a region of long flagella at the posterior pole and its spicules are very long (ca. 130 μ). In fact, despite the apparent differences in oogenesis and early embryonic development, the larva of *Halichondria* sp. most closely resembles that of *Halichondria coalita* (Topsent, 1911; Meewis, 1941). It is also similar to the larva of *Halichondria boverbanki*, except that the latter bears the longest flagella at the anterior pole (Hartman, 1958).

The reproductive period of *Halichondria* sp. was analyzed using different techniques which, when considered separately, seemed to suggest somewhat different conclusions. Microscopic analysis of postdormant specimens indicated that the reproductive period extends from May through July, with the peak of reproductive activity occurring in June. On the other hand, observation of larvae in the plankton showed that the reproductive period extends at least into August, and a study of larval settlement on clam shells suspended in the Mystic Estuary suggested that the peak of reproductive activity occurs during that month. Subsequent microscopic examination of postlarval specimens showed that they become reproductive in July and continue to reproduce until at least mid-October. It therefore appears that settlement on the clam shells was primarily by larvae produced by postlarval specimens which are frequently very abundant at this time of year. Evidently, there is sequential reproduction by postdormant and postlarval specimens with some overlapping of reproductive periods in July. Although there is no specific information available on this point, it is possible that two or more generations of reproductive postlarval specimens may be produced during the summer and fall.

Halichondria sp. begins reproductive activity very soon after emerging from winter dormancy. In this respect, it resembles *Haliclona loosanoffi* (Fell, 1974b, 1976a), *Microciona prolifera* (Simpson, 1968), *Ephydatia fluviatilis* (Van de Vyver and Willenz, 1975) and *Spongilla lacustris* (Simpson and Gilbert, 1973; Gilbert, 1974). In both *Haliclona loosanoffi* (Fell, 1976b) and *Ephydatia fluviatilis* (Van de Vyver, 1970; Van de Vyver and Willenz, 1975) it appears that only specimens which have undergone winter dormancy become reproductive. *Haliclona loosanoffi* (in New England) overwinters only in the form of gemmules, but *Ephydatia fluviatilis* (in Belgium) survives the winter both as gemmules and as dormant sponge tissue lacking flagellated chambers. In the latter case, it appears that only sponges developing from dormant tissue become sexually reproductive; those developing from gemmules do not (Van de Vyver, 1970).

Few sponges, besides *Halichondria* sp., have been definitely shown to exhibit sexual reproduction in postlarval specimens during the same year in which they

are produced, although this may be a fairly widespread phenomenon. Gametogenesis and embryonic development were observed in postlarval specimens of an unidentified fresh-water sponge and low levels of egg production were noted in postlarvae of *Spongilla lacustris* (Simpson and Gilbert, 1974). However, in the latter case, it is unclear whether breeding takes place, since neither sperm nor larvae were observed. Postlarval specimens of *Haliclona loosanoffi* may contain a few small oocytes or spermiatic cysts but not large oocytes, embryos or larvae (Fell, 1976b).

The fact that postlarval specimens of *Halichondria* sp. reproduce during a period when postdormant specimens do not suggests that environmental conditions are suitable for sexual reproduction over a relatively long time and that the reproductive periods of postdormant and postlarval specimens are, to a large extent, under endogenous control. This conclusion is also consistent with the findings of Gilbert (1974) concerning the regulation of reproduction in *Spongilla lacustris*. The normal reproductive period of this sponge extends from late April to late June (in New Hampshire). However, when gemmules are implanted back into the pond of origin at any time between late spring and early fall, they germinate into sponges which begin to develop oocytes in about 1 week. The production of new oocytes usually continues for only three to four weeks. It therefore appears that, following a short period of sexual reproduction, the physiology of the sponge changes in some way so that further sexual reproduction can no longer occur. This change might involve the exhaustion of nutritional reserves, among other things (Gilbert, 1974). On the other hand, environmental regulation of the sexual reproductive period may predominate in populations of *Ephydatia fluviatilis* in Belgium (Van de Vyver and Willenz, 1975).

The larvae of *Halichondria* sp. frequently settle gregariously, with many larvae settling within a restricted area. Especially when larval settlement is heavy, fusion of adjacent specimens may occur, leading to the formation of genetic mosaics. Field studies by Burton (1949) suggest that such larval fusion also occurs in *Halichondria panicea* and *Hymeniacidon perlevis*, as well as in several other species, under natural conditions. Furthermore, fusion of larvae and/or postlarvae in the laboratory has been described for a number of sponges, including *Mycale syrinx* (Wilson, 1935), *Tethya aurantium* (Lévi, 1956), *Polymastia robusta* (Borojević, 1967), *Ophlitaspongia seriata* (Fry, 1973), *Ephydatia fluviatilis* (Van de Vyver, 1970), and *Haliclona loosanoffi* (Fell, 1976a). It therefore appears that larval and/or postlarval fusion may be fairly common among sponges. In the fresh-water sponge, *Ephydatia fluviatilis*, larvae produced by specimens of the same strain fuse to form single sponges, while the cells of larvae derived from different strains segregate following initial larval fusion (Van de Vyver and Willenz, 1975). The formation of genetic mosaics by the fusion of larvae and/or postlarvae is an interesting phenomenon which may be the basis of the incomplete gonochorism found in this and other sponges.

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SUMMARY

1. A description of gametogenesis and embryonic development in *Halichondria* sp. is presented.
2. *Halichondria* sp. exhibits an incomplete gonochorism in which some specimens produce either eggs or sperm and others produce both. When the sexes are separate, the sex ratio is about 1:1.
3. In the Mystic Estuary, Connecticut, the reproductive period of postdormant specimens extends from May into July, while the reproductive period of postlarval specimens begins in July and continues at least into October. Since the reproductive periods of postdormant and postlarval specimens are separated, it appears that reproduction is, to a large extent, under endogenous control.

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LARVAL DEVELOPMENT OF *PAGURUS HIRSUTIUSCULUS* (DANA) REARED IN THE LABORATORY

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The hairy hermit crab, *Pagurus hirsutiunculus* (Dana) has an extensive North Pacific distribution. It ranges from Siberia, the Sea of Okhotsk of U.S.S.R. and Northern Japan, to the west coast of North America from the Pribilof Islands in the Bering Sea south to Southern California, and from the intertidal zone to 110 m (McLaughlin, 1974). It is one of the most common hermit crabs in upper intertidal zones of Puget Sound and Washington coastal beaches. Of the 180 described species of *Pagurus* (Gordon, 1956), 25 have been described in N.W. North America (McLaughlin, 1974), and 18 have been identified in Washington waters (Kozloff, 1974). Published larval descriptions include 21 species of *Pagurus*, of which only two are reported from Washington waters.

This paper describes the external anatomy of five *Pagurus hirsutiunculus* larval stages: four zoea and a glaucothoe, as raised in the laboratory. Five sibling sets of larvae are compared as to durations of the four zoeal stages, anatomical variations, number of eggs hatched, and the size of the females. The effects of varying day lengths on growth and mortality of zoeal stages are also presented, using zoea from three sibling sets. The place of this species in the groupings of larvae suggested by MacDonald, Pike and Williamson (1957), Pike and Williamson (1960), and Roberts (1970) is discussed. Comparisons are made with California specimens of *P. hirsutiunculus* (Hall, unpublished, 1972).

MATERIALS AND METHODS

Collections of adult *Pagurus hirsutiunculus* were made from Salt Water State Park (47°22'30" N, 122°19'30" W) and from Reservation Bay, Deception Pass State Park, Fidalgo Island (48°24'50" N, 122°39'10" W) in Puget Sound, Washington, at the 2 to 4 foot tide level, during May and June, 1976. Ovigerous females were placed in a laboratory refrigerator at 13° C ($\pm 2^\circ$) in individual, foil-covered quart jars of unfiltered sea water and observed daily.

Upon hatching, the larvae were placed in the same 13° C refrigerator in quart jars of sea water filtered through 64 μ m sieves. A sibling set from one female (Set A, Table I) was cultured at 10 larvae per jar, and sets B, C, D, and E (Table I) initially at 50 larvae per jar. A 15 watt incandescent bulb provided constant light.

Larvae were fed daily with freshly hatched *Artemia salina* nauplii as Forss and Coffin (1960) described. Observations for stage changes, numbers, coloration and behavior were made daily. The larvae were transferred every 2 days to freshly filtered water collected from Puget Sound every 4 days, and stored at 13° C. Salinity was not held constant.

TABLE I

Mean duration in days with (ranges) of five sibling sets of *Pagurus hirsutiunculus* larvae through four zoeal stages, including correlations of size of female (carapace length in mm), number of eggs hatched, and hatching time in hours.

Zoeal stage	Sibling set					Mean	Median
	A	B	C	D	E		
I	10.8 (10-12)	11.3 (11-12)	9.6 (9-10)	9.4 (9-10)	8.5 (8-9)	9.9 (8-12)	9.4
II	10.2 (10-12)	8.9 (8-9)	10.0 (10)	10.1 (10-11)	10.0 (10)	9.8 (8-12)	9.6
III	8.0 (8)	7.7 (7-8)	9.0 (9)	8.3 (8-9)	8.0 (8)	8.2 (7-9)	7.5
IV	9.5 (7-12)	9.9 (7-13)	12 NM	10.7 (6-15)	10.0 (7-14)	10.0 (6-15)	9.0
Total	37.5 (35-41)	35.9 (33-39)	40.0 (40)	37.7 (33-42)	36.2 (34-41)	36.3 (33-42)	35.5
							Correlation Coefficients
Egg No.	168	268	106 (322)*	660	461		1.0000
Size ♀ (CL in mm)	7	9	10	14	13		.9655
Hatch time (hours)	48	36	48	3	24		-.9375

NM = Never molted, died at 12.

* Includes discarded eggs which hatched.

In a separate experiment, gravid crabs were collected from Reservation Bay. Upon hatching, the zoeae were separated into quart jars and placed in photoperiod chambers using light: dark cycles of 24:0, 21:3, 18:6, 15:9, 12:12, 9:15, 6:18, 3:21, and 0:24 hr. The 0:24 cycle had about 2 minutes of light each day for observation and culture techniques. Two sibling sets of 270 zoeae, 10 zoeae per jar and 6 jars in each chamber, were distributed equally in one experiment and one set of 540 siblings were used in a second experiment. The studies were otherwise conducted under conditions similar to those described above, and took place June through August, 1966. Observations as to stage changes and mortality were made daily. The mean zoeal stage was determined when 50% of the zoea had molted to the next stage.

Statistical comparison between sibling sets of larvae from the varying photoperiod groups utilized a BMD linear regression computer program which arrived at regression coefficients expressing the daily mean zoeal stage of larvae in a group *vs.* the time in days since the commencement of the culturing. The regression coefficients were compared for significant differences using Ostle tests. Daily mortality was also plotted *vs.* time in days and analyzed in the same way. All correlation coefficients were determined from the correlation analysis of the Cosap computer program.

Of the 1664 larvae from five females originally cultured, 90 were preserved in glycerine, dissected with minuten needles, and mounted on glass slides in Hoyer's mounting medium. A minimum of two specimens from each sibling set at each stage were completely dissected and mounted. All were compared for anatomical variations and measurements. Drawings were made from sibling set D (Table I).

The number of eggs hatched was determined by live zoeae counted upon removal from the jar with the female. The duration of stages was identified as the time spent in a given instar by larvae which successfully survived molting to the next stage.

Drawings and measurements were done with a Leitz phase contrast microscope equipped with camera lucida and ocular micrometer. Living larvae and those freshly killed in glycerin were measured and observed for chromatophore patterns.

The terminology used in descriptions follows that of Nyblade (1970). Abbreviations used are as follows A1 = antennule, A2 = antenna, Mn = mandible, Mx1 = maxillule, Mx2 = maxilla, Mxp1 to Mxp3 = first to third maxilliped, P1 to P5 = first to fifth pereopod, P12 to P15 = second to fifth pleopod, U = uropod, N = number observed, TL = total length, CL = carapace length.

Total length was measured from the tip of the rostrum to the posterior border of the telson, excluding the telson processes. Carapace length was measured from the tip of the rostrum to the most posterior lateral margin of the carapace, including the postero-lateral carapace spines. Measurements of lengths were plus or minus 0.1 mm. The numbering of the telson processes follows Pike and Williamson (1960).

RESULTS

Four zoeal stages and a glaucothoe were observed. No post hatching prezoaea were present in any of the five sibling sets studied. The average length of time from hatching through final molt to glaucothoe was 36.3 days, median 35.5 days (Table I). Descriptions of Zoea II through Zoea IV include only changes from the previous zoeal description. The glaucothoe is completely described.

Zoea I

Duration: 8 to 12 days, mean 9.9 days (N = 471) at 13° C.

Size: TL = 2.4 mm (N = 20), CL = 1.4 mm (N = 20).

Description (Fig. 1A, E): Carapace without processes except projections on postero-lateral margins. Eyes sessile. Rostrum long, tapering to thin point, slightly longer than antennae. Abdomen of five distinct somites and sixth fused to telson. Somite one lacks spines, two to five bear three pair dorso-lateral spines, no apparent differences in spine lengths. Anal spine present. Pleopod buds and uropods absent.

Immediately upon hatching, numerous yellow chromatophores visible around eyes and over carapace and abdomen, but not apparent after 24 hr. Only red chromatophores present later, uniform in location, size and intensity within sibling sets, varying in size and intensity between sibling sets. Most star-shaped but irregular. Two at base of antennae and antennules between eyes, two more immediately posterior to eyes. Two large on lateral sides of carapace with longer weblike extensions surrounded by diffuse red. One located immediately anterior

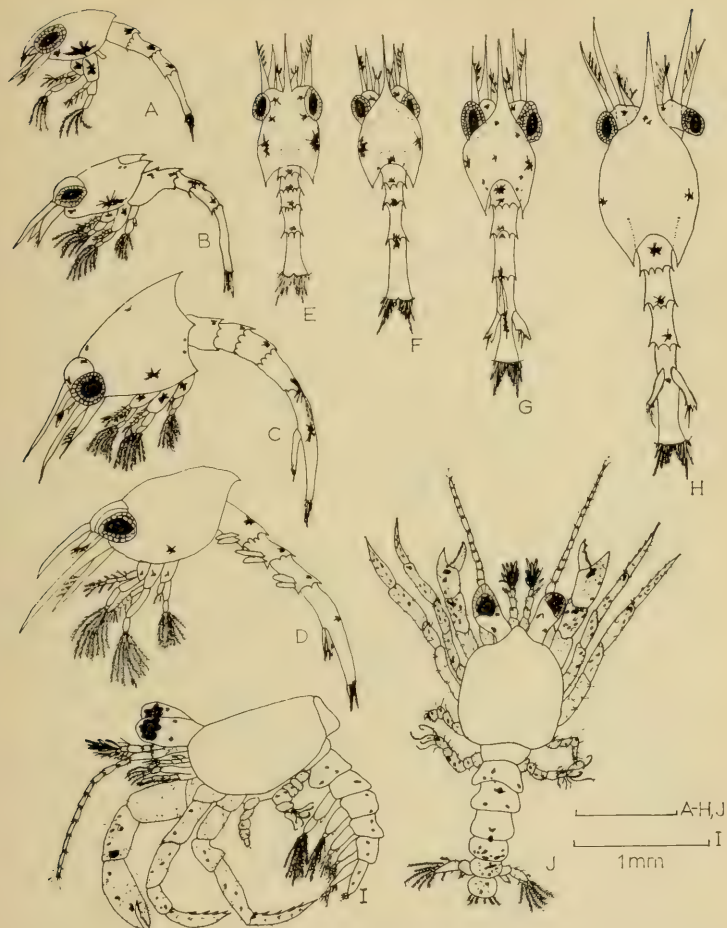


FIGURE 1. Whole mount *Pagurus hirsutiussculus* larvae. Lateral view: Zoea I (A), II (B), III (C), IV (D), glaucothoe (I). Dorsal view: Zoea I (E), II (F), III (G), IV (H), glaucothoe (J).

to dorsal spines on somites two through five, the one on somite five projecting posteriorly into somite six. Two located on base of mandibles, one on each protopodite of Mxp1 and Mxp2 and on exopodite of Mxp1. Two diffuse yellow areas located

medially and anterior to lateral carapace red chromatophores. Eyes an iridescent yellow-green.

A1 (Fig. 2A): Uniramous; unsegmented; two long, terminal aesthetascs, shorter of smaller diameter; three short, terminal simple setae; one long, subterminal seta with setules.

A2 (Fig. 2E): Biramous, scale with four setae with setules and one smaller, simple seta on inner margin plus terminal spine. Scale including spine $8 \times$ maximum width, equal in length to endopodite. Endopodite nude, protopodite one short, stout, serrated spine on distal end.

Mn (Fig. 2I): Asymetrical with numerous teeth, wide variability, no palp.

Mx1 (Fig. 2N): Coxal endite with five large setae with setules terminally and one small, simple seta subterminally. Basal endite with two stout teeth bearing spinules and two simple setae at base. Endopodite of three segments; second with one long, simple seta on medial margin; terminal with three long, simple setae.

Mx2 (Fig. 2S): Coxal endite bilobed, distal lobe with three terminal, simple setae and one subterminal, long, simple seta; proximal lobe with four terminal, long, simple setae. Basal endite bilobed, distal lobe with two terminal and one subterminal, long setae with setules; proximal lobe with two terminal, simple setae, one subterminal, long seta with setules, and one short simple seta. Endopodite with three terminal and one subterminal, long setae with setules. Proximal lobe with two long setae with setules and one short, simple seta. Scaphognathite with five plumose setae along margin.

Mxp1 (Fig. 3A): Basipodite with two setae with setules and one simple seta on distal lateral margin, seven short, simple setae scattered down lateral margin. Endopodite of five segments: first with two long, one short setae with setules on lateral margin; second with two long setae with setules on distal lateral margin; third with one long seta with setules on distal lateral margin; fourth with two long setae with setules on lateral margin, one on medial margin; terminal with four terminal, long setae with setules. Number of long hairs on medial margin of segments one through three. Exopodite partially segmented with four long, two-segmented, plumose, natatory setae.

Mxp2 (Fig. 3E): Basipodite with one simple seta and one barbed seta on distal lateral margin and one short, hairlike, simple seta in center of segment. Endopodite of four segments: first and second segments with one seta with setules and one barbed seta each on distal lateral margin; third with one seta with setules on distal medial margin and one seta with setules and one barbed seta on distal lateral margin; terminal segment with four terminal, long setae with setules. Exopodite of two segments, terminal one with four very long, two-segmented, plumose, natatory setae.

Mxp3 (Fig. 3I): Uniramous, partially segmented rudiment with one terminal papilla.

Telson (Fig. 3M): Longer than wide, with longest spine $1.2 \times$ telson width. Posterior margin bilaterally convex with V-shaped notch in center and seven processes on each side, all articulated. Outer simple spine (1); hairlike simple seta (2); articulated spines plumose on medial margin with several rows

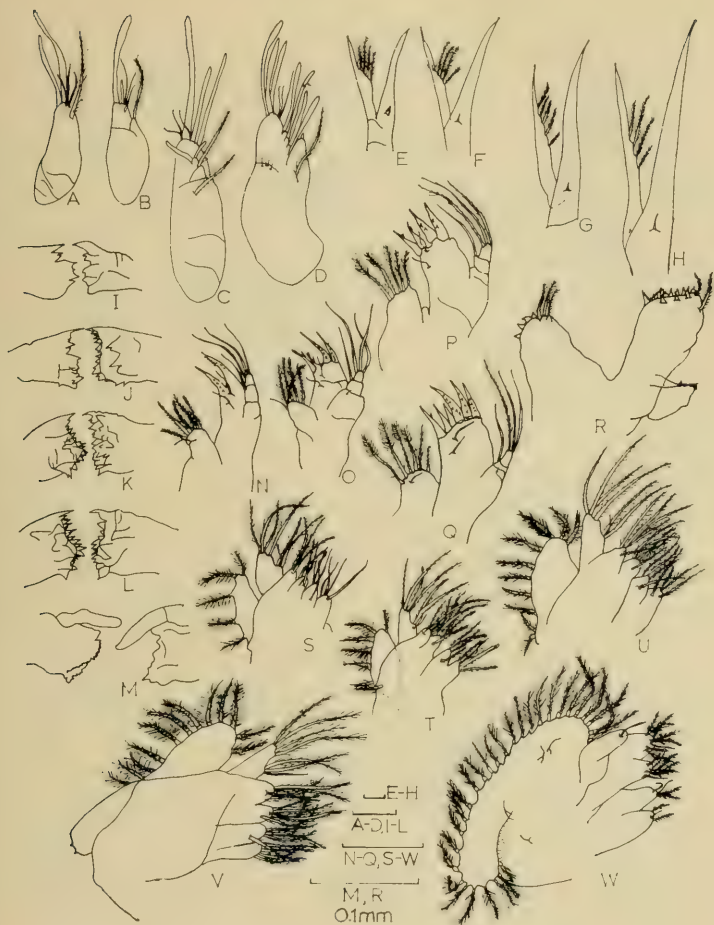


FIGURE 2. Appendages of *Pagurus hirsutiussculus* larvae: antennule Zoea I (A), II (B), III (C), IV (D); antenna Zoea I (E), II (F), III (G), IV (H); mandibles Zoea I (I), II (J), III (K), IV (L), glaucothoe (M); maxillule Zoea I (N), II (O), III (P), IV (Q), glaucothoe (R); maxilla Zoea I (S), II (T), III (U), IV (V) glaucothoe (W).

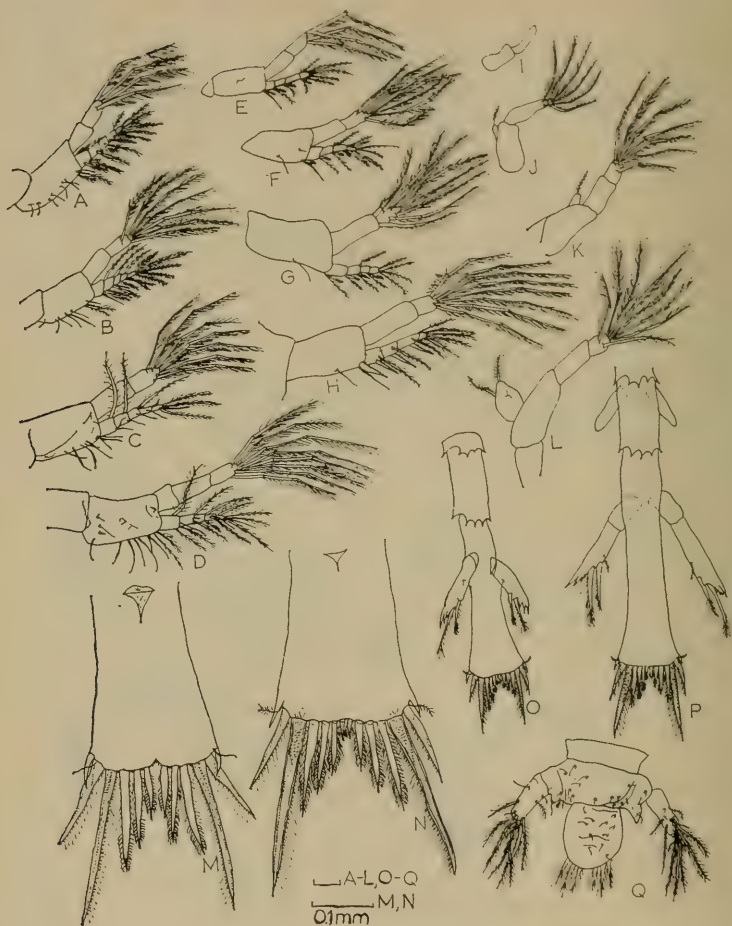


FIGURE 3. Appendages of *Pagurus hirsutiusculus* larvae: first maxilliped Zoea I (A), II (B), III (C), IV (D); second maxilliped Zoea I (E), II (F), III (G), IV (H); third maxilliped Zoea I (I), II (J), III (K), IV (L); telsons Zoea I (M), II (N), III (O), IV (P), glaucothoe (Q).

of tiny spinules (3, 4), heavily plumose at sharp pointed tip and lighter proximally (5-7).

Zoea II

Duration: 8 to 12 days, mean 9.8 days (N = 195) at 13° C.

Size: TL = 2.6 to 2.8 mm (N = 20), CL = 1.4 mm (N = 20).

Description (Fig. 1B, F): Eyes stalked. Rostrum heavier than in Zoea I but same length, as is carapace. Relationship and sizes of antennae, antennules and rostrum same. Abdomen with only four somites visible dorsally posterior to carapace, two more underneath. Somite five with two pair dorso-lateral spines; three and four same as Zoea I; one and two with no spines.

Chromatophores identically located with exception of one posterior to eyes absent. Four red spots present medial to carapace spines.

A1 (Fig. 2B): One long, terminal aesthetasc; one short. Shorter seta with setules subterminally now on palp.

A2 (Fig. 2F): One small, additional hair at location of simple seta on scale. Endopodite slightly longer than scale.

Mn (Fig. 2J): Increase in size and number of teeth, variability in individuals.

Mx1 (Fig. 2O): Two additional, slender teeth with spinules on basal endite with two simple setae proximal to two smaller teeth near inner margin.

Mx2 (Fig. 2T): Both distal and proximal lobes of basal and coxal endites now with three terminal and one subterminal setae with setules. Scaphognathite has seven plumose setae along margin.

Mxp1 (Fig. 3B): Basipodite with reduction to six simple setae, grouped 3, 2, 1 down margin. Endopodite segments one to three each with one long seta with setules on distal medial margin replacing hairs. Exopodite with seven three-segmented, natatory, plumose setae.

Mxp2 (Fig. 3F): Basipodite one additional, simple seta on lateral margin. Endopodite one additional, long seta with setules on medial margin of segment two. Exopodite with seven three-segmented, plumose, natatory setae.

Mxp3 (Fig. 3J): Endopodite a small, conical projection with one short seta with setules. Exopodite one segment with six three-segmented, plumose, natatory setae, shorter than on Mxp1 and Mxp2.

Telson (Fig. 3N): Eight processes on each side, added pair medial and shortest of all. Spine fused (1), with the rest articulated. V-shaped notch no longer present. Longest process $1.3 \times$ telson width.

Zoea III

Duration: 7 to 9 days, mean 8.2 days (N = 106) at 13° C.

Size: TL = 3.2 mm (N = 20), CL = 1.7 mm (N = 20).

Description (Fig. 1C, G): Rostrum longer than in Zoea I, II. Antennae slightly longer than rostrum. Sixth abdominal segment incompletely fused to telson.

Additional red chromatophore at border of sixth somite and telson, with hair-like extensions anterior joining large chromatophore on somite five. Lateral

carapace chromatophores smaller, with addition of numerous red spots placed irregularly on carapace. Yellow-green, indefinitely bounded areas present posterior and medial to eyes.

A1 (Fig. 2C): Biramous, segmented. Three large, terminal aesthetascs plus three simple setae; two smaller aesthetascs subterminally; one seta with setules on proximal segment.

A2 (Fig. 2G): Distal seta on scale now with setules, proximal seta shorter than in *Zoea* II.

Mn (Fig. 2K): Increase in size and number of teeth.

Mr1 (Fig. 2P): Coxal endite with outer portions of four terminal setae with heavier setules, loss of simple seta. Two of terminal setae on endopodite now with fine setules.

Mr2 (Fig. 2U): Proximal and distal lobes of basal endite with four terminal and one subterminal setae with setules. Scaphognathite with 10 plumose setae.

Mrp1 (Fig. 3C): Basipodite with nine setae, all simple, grouped 3, 2, 1, 2, 1. Endopodite terminal segment with three setae with setules and one simple seta; one very long seta with setules on distal medial margin of each of segments one through three; short, hairlike, simple seta on segment four. Setae on lateral margin of segments all simple; two on first, one each on segments two through four.

Mrp2 (Fig. 3G): Basipodite with no simple seta in center; seta on distal lateral margin now with setules. Endopodite, no seta with setules on segment three medial margin. Exopodite with eight plumose, natatory setae.

Mrp3 (Fig. 3K): Exopodite two-segmented with seven plumose, natatory setae.

Telson and U (Fig. 3O): Uropods each with two long, terminal spines, two large spines with spinules and one simple seta proximally. Telson width and length of all telson spines increased.

Zoea IV

Duration: 6 to 15 days, mean 10.0 days ($N = 43$) at 13° C.

Size: TL = 4.1 mm ($N = 20$), CL = 2.3 mm ($N = 20$).

Description (Fig. 1D, H): Pleopod buds on abdominal somites two through five. Eyestalks more elongated laterally. Reduction in size of all chromatophores, especially on carapace and base of antennae. Two red spots at base of rostrum.

A1 (Fig. 2D): Four aesthetascs subterminally. No seta on terminus of peduncle; basal seta longer than on *Zoea* III. Four simple setae in row at base of distal segment.

A2 (Fig. 2H): Spine present at base of endopodite.

Mn (Fig. 2L): More increase in size and number of teeth.

Mr1 (Fig. 2Q): Coxal endite with six setae with setules, heavier than in *Zoea* III; one simple seta. Basal endite with six teeth bearing spinules; two simple setae. Endopodite with tiny, hairlike seta on proximal segment.

Mr2 (Fig. 2V): Coxal endite lacks simple seta on proximal lobe. Scaphognathite with 12 plumose setae on main lobe; a second naked lobe with two tiny papillae.

Mxp1 (Fig. 3D): Basipodite with two setae with setules and seven simple setae scattered along margin; three simple setae near base, spine in center of segment. Endopodite back to setation similar to *Zoea* II, except one seta on each of segments four and five, simple; short seta with setules on distal medial margin of segment four. Exopodite with eight plumose, natatory setae; spine at base.

Mxp2 (Fig. 3H): Basipodite two simple setae plus barbed seta. Endopodite with loss of seta on medial margin of segment two, present on three; additional small hair at base of segments one through three; barbed setae on segments one through three and basipodite, much longer than in other zoea.

Mxp3 (Fig. 3L): Endopodite with two long, subterminal setae with setules; one short, simple seta. Exopodite with eight plumose, natatory setae.

Telson and U (Fig. 3P): Exopodite of uropod articulated with protopodite. One long, three shorter spines on terminal end of exopodite. Two, heavily plumose, long setae and one small, plumose, hairlike seta proximally on exopodite medial margin. Telson longer; width, process length same.

Glaucothoe

Duration: Undetermined.

Size: TL = 2.8 mm (N = 43), CL = 1.3 mm (N = 43).

Description (Fig. 1I, J): Rostrum reduced and rounded; no postero-lateral spines on carapace. Antennae two-thirds the length of combined carapace plus abdomen-telson. Pereiopods and paired pleopods present and functional. Red chromatophores present on eyestalks, pereopods 1 to 5, abdominal segments and telson, mostly as deeply pigmented spots. Yellow chromatophores on second and terminal segments of P1 and fourth segment of P2. Entire larva a pale, translucent blue with increased intensity of color on eyestalks and A1 to A2 bases. Abdomen with six unequally sized and shaped somites.

A1 (Fig. 4A): Outer ramus of four segments: second with four long aesthetascs, third with one long and four short, simple setae on distal margin, plus four long aesthetascs; terminal with one long and three short, simple setae terminally, three long aesthetascs. Inner ramus of two segments: first with two simple setae on distal margin; terminal with three simple setae terminally and five simple setae subterminally. Peduncle of three segments: first with three simple setae; second with five simple setae on distal margin and one spine medially between setae, two simple setae at base; distal with four simple setae.

A2 (Fig. 4B): Scale with three simple setae. Flagellum of 12 to 14 segments, each with 4 to 6 simple setae except the terminal (3) and proximal (0). Peduncle of five segments, each with 2 to 4 simple setae except for basal segment (0).

Mn (Fig. 2M): Smaller than *Zoea* I. Teeth much reduced in size and number. Incompletely segmented, naked palp extending well past tooth surfaces.

Mx1 (Fig. 2R): Coxal endite with four tooth-like spines and three short setae with setules on terminal end. Basal endite with 14 larger, tooth-like spines, some serrated, plus one short seta with setules terminally, one additional short plumose seta proximally. Endopodite with five very small, tooth-like spines.

Mx2 (Fig. 2W): Coxal endite bilobed: distal lobe with two setae with setules and two simple setae terminally; proximal lobe with two setae with setules and



FIGURE 4. Appendages of *Pagurus hirsutiunculus* at glaucothoe stage: antennule (A), antenna (B), first maxilliped (C), second maxilliped (D), third maxilliped (E), left cheliped [first pereiopod] (F), right cheliped (G), second and third pereiopods (H), fourth pereiopod (I), fifth pereiopod (J), second pleopod (K), third pleopod (L), fourth pleopod (M), fifth pleopod (N).

one simple seta terminally. Basal endite bilobed: distal lobe with seven setae with setules and one simple seta terminally; proximal lobe with four setae with setules and two simple setae terminally. Endopodite with one simple seta terminally, one simple seta at base of endites. Scaphognathite with 28 to 30 plumose setae along margin plus a number of small hairs at either end of lobe, two simple setae in center. Naked lobe found in Zoa IV absent.

Maxp1 (Fig. 4C): Coxa with two simple setae on margin, basipodite broad, with ten simple setae on margin, three simple setae near base. Endopodite partially two-segmented with two simple setae subterminally. Exopodite partially two-segmented with four small, simple setae terminally, one seta subterminally.

Maxp2 (Fig. 4D): Endopodite of four segments with one simple seta on third, four simple setae on terminal segment. Exopodite of two segments with four long, three-segmented, plumose setae; two shorter, unsegmented setae with setules terminally, one small, simple seta subterminally.

Maxp3 (Fig. 4E): Number of hairs on coxa. Endopodite of five segments, longer than exopodite. First segment (basis-ischium fused) with four simple setae and a few hairs; toothlike crista dentata near distal end. Second segment with one seta with setules, three simple setae. Third segment with three setae with setules, three simple setae. Fourth segment with nine setae with setules, seven of which have heavy hairs, two with fine hairs; six simple setae. Terminal segment with eight setae with setules, of which two have heavy hairs, two have very fine hairs; two simple setae. Exopodite of three segments, proximal segment with one simple seta, terminal segment with five long, three-segmented, plumose setae; three short, simple setae.

P1 (Fig. 4F, G): Right slightly larger than left. Three segments; over one-half the length of A2. Merus with 16 to 18 simple setae dorsally; carpus with 12 to 14 simple setae dorsally; chela with 40 to 45 setae dorsally. Dactyl exceeding length of fixed finger with jagged, tooth-like projections on cutting edge of dactyl.

P2 and P3 (Fig. 4H): Nearly identical in size, shape, setation. Ischium with six simple setae; merus with 13 simple setae; carpus with 11 to 12 simple setae; propodus with three spines and 13 to 14 simple setae, all dorsally. Dactyl a single claw with 4 spines and 19 to 22 simple setae.

P4 (Fig. 4I): Subchelate. Basis with four simple setae; ischium with seven simple setae; merus with eight simple setae; carpus with three simple setae; propodus with four corneous tubercles along lateral margin plus three simple setae, two simple setae on opposite margin. Dactyl claw-like, two corneous tubercles, five simple setae, one small hair.

P5 (Fig. 4J): Subchelate. Basis with five simple setae; ischium with three simple setae; merus with four simple setae. Carpus with one very long, curling seta with short hairs on terminal half only, four simple setae. Propodus with five corneous tubercles, two tooth-like tubercles, six long, curling setae with short hairs, and five simple setae. Dactyl with four corneous tubercles plus three simple setae.

P1 2 (Fig. 4K): Endopodite one-third the size of exopodite, two minute hooks on terminal end; hooks are identical in P1 2-5. Exopodite with eight long, unsegmented, plumose, natatory setae; one shorter, plumose seta proximally.

P1 3 (Fig. 4L): Exopodite with seven long, plumose, natatory setae, and one shorter, plumose seta.

P1 4 (Fig. 4M): Exopodite with eight plumose, natatory setae.

P1 5 (Fig. 4N): Exopodite with six plumose, natatory setae. Pleopods decrease in size in somites two through five.

Telson and U (Fig. 3Q): Telson with six setae with setules on posterior margin and five pair of simple setae spaced on dorsal surface. Uropod protopodite with three simple setae. Endopodite with two simple setae, two corneus tubercles. Exopodite with six long setae with setules (four terminal, two subterminal), two shorter setae with setules proximally, five corneus granules and four simple setae terminally, plus two simple setae proximally. Left uropod longer than right and less sharply defined between exopodite and protopodite.

Variation

Anatomical variations between sibling sets were minor. Zoea II of one sibling set was 0.1 mm shorter in total length than the other four sets, but all other measurements taken on a total of 123 larvae were within 0.1 mm of each other throughout. The mandible was the only appendage showing variation in the four zoeal stages. Here each individual appeared to have a different tooth pattern, although sizes were consistent. Individuals used for the drawings of the mandibles (Fig. 2, I-M) were representative.

In the glaucothoe stage there was variation in the number of natatory setae on the five pairs of pleopods that did not appear to be due to breakage. Setation also varied on pereopods one to three.

There was evidence of breaking and regrowth of setae on the first and second maxillipeds of all zoeal stages, the new growth being visible internally. Chromatophore patterns varied for the first 24 hr after hatching and in the glaucothoe, but were consistent for the remaining stages. There was no increase in size or length of zoea between molts.

Although all zoeal stages exhibited positive phototaxis, variations in the 24-hr light:dark cycles did not show a significant effect on molting rate and survival of larvae. Treatment regression coefficients for growth (molting) and mortality rates are shown in Table II. Results for both rates were not significantly different at the 0.05 level. No trend due to the light:dark treatments was evident.

DISCUSSION

P. hirsutiunculus is one of the two species of *Pagurus* most abundant in upper tidal Washington waters, but the larval stages had not been described or categorized with other *Pagurus* larvae. Larvae of 21 other species have been at least partially described in the literature since 1937, with only 9 of these complete for the 5 larval stages. A list is found in Nyblade and McLaughlin (1975) and Fitch (unpublished master's thesis, University of Puget Sound, 1977). Among those described *P. hirsutiunculus* larvae appear to show the most similarity to *P. samuelis* (MacMillan, 1971), as do the adults of the two species (McLaughlin, 1974).

TABLE II

Relative growth (molting rate) and mortality rate of three summed sibling groups of *Pagurus hirsutiunculus* larvae in light:dark treatments during the first 27 days of development. Regression coefficients (β), standard error of the regression coefficients ($S\beta$) and correlation coefficients (r) for growth plotting daily mean zoeal stage vs. time in days; mortality rate plotting numbers of zoea surviving vs. time in days.

Treatment Light:dark (hours)	Growth Rate			Mortality Rate		
	β	$S\beta$	r	β	$S\beta$	r
24:0	0.123	0.003	0.991	3.139	0.161	0.970
21:3	0.128	0.004	0.990	3.154	0.186	0.960
18:6	0.132	0.004	0.988	2.412	0.122	0.970
15:9	0.125	0.003	0.992	2.934	0.200	0.948
12:12	0.132	0.005	0.981	1.582	0.073	0.976
9:15	0.132	0.004	0.990	2.883	0.212	0.941
6:18	0.133	0.004	0.990	2.526	0.178	0.945
3:21	0.135	0.004	0.990	3.386	0.300	0.917
0:24	0.133	0.004	0.989	3.139	0.161	0.970

Four groupings of *Pagurus* larvae, based on a number of characteristics, are presented in Roberts (1970), MacDonald *et al.* (1957), and Fitch (unpublished, 1977). Recent descriptions of *Pagurus* larvae do not fit exactly into any of the four groups. *P. hirsutiunculus* best fits in Group A, in 14 out of the 17 characteristics. Of those differing, the length of the lateral spines on somite five (medium in this species) is questionable as a distinguishing characteristic. The mandibular palp, although absent in Zoea IV, is present in the glaucothoe. The most significant difference from Group A is in the fourth telson process of Zoea III and Zoea IV, which is articulated, not fused, in all stages of this species. *P. samuelis* has been tentatively placed in Group A (Roberts, 1970), and it also has an articulated fourth telson process here (MacMillan, 1971). Articulation or fusion of the telson processes may be a useful characteristic for keying *Pagurus* larvae, as it is quite easily observed in all zoeal stages.

Chromatophore patterns (characteristics listed in the groupings) appear unreliable for distinguishing purposes, based on observations with this species. Plankton specimens were not used for comparison in this or most other larval descriptions, thus the groupings appear to have little identification value to date. As suggested by Nyblade (1970), the present groupings have questionable value overall. No other groupings are made obvious by comparing *Pagurus* larval descriptions.

Table III shows total body length of larval stages from descriptions of 19 *Pagurus* species. There appears to be no correlation with regard to size when species are placed in the original groupings. The genus appears to have a wide range of larval sizes with a high standard deviation. Twelve species (starred in Table III) do appear to show size similarity throughout the five larval stages. They include three from Group A, four from Group B, one from Group D and the other four uncategorized. Size appears inappropriate for grouping of larvae and may be of little value in identifying them from the plankton. Laboratory reared specimens appear to be smaller than planktonic ones (Roberts, 1973), and the same

species described by different authors (*P. hirsutiunculus*, *P. samuelis*, and *P. prideauxi*, Table III), show a variation in size within the laboratory cultured specimens.

A comparison of this study with an unpublished master's thesis study done by W. R. Hall at the University of the Pacific, California, in 1972 on *P. hirsutiunculus*, indicates that most variation within the species occurs in duration of stages and zoeal size. There was an 11.3 day increase in mean number of days from hatch to glaucothoe in the Washington study (36.3 vs. 25 days). Durations of individual stages also showed increases, from 1.7 days in Zoea III to 4.8 days in Zoea II. There was a culture temperature difference of 3° C (13° C Washington vs. 16° C California), as well as photoperiod, salinity and other possible cultural differences which may account for the variation. The 13° C temperature corresponds to the average summer temperatures of Puget Sound; California waters would be warmer. If the 13° C temperature approaches the lower thermal limit and 16° C is closer to optimum, this could account for stage duration changes. A further study is in preparation to determine the effects of thermal variation on stage duration.

TABLE III

Comparisons of sizes of zoea, total length in mm, for the Pagurus species described.

Species	Researcher	Zoea				Glaucothoe
		I	II	III	IV	
<i>acadianus</i> , Roberts (1973)		3.0	3.7	4.6	5.6	3.1
<i>alatus</i> , Bookhout (1972)		3.3	3.9	4.4	5.3	3.4
<i>annulipes</i> ,* Nyblade (1970)		2.2	2.6	3.0	3.8	2.9
<i>beringanus</i> ,* Hart (1937)		2.4	2.9	3.2	3.6	1.9
<i>bernhardus</i> , MacDonald <i>et al.</i> (1957)		3.6	5.0	6.5	8.2	3.7
<i>cuanensis</i> ,* MacDonald <i>et al.</i> (1957)		2.7	2.9	3.6	3.9	2.9
<i>exilis</i> ,* Scelzo and Boschi (1969)		2.5	3.4	3.2	4.0	1.3
<i>granosimanus</i> ,* Hall (unpub., 1972)		2.1	2.4	3.1	3.6	2.4
<i>hemphilli</i> ,* Hall (unpub., 1972)		2.1	2.4	3.0	3.4	2.1
<i>hirsutiunculus</i> ,* Hall (unpub., 1972)		1.9	2.5	3.1	3.6	2.3
<i>hirsutiunculus</i> ,*		2.4	2.6	3.2	4.1	2.8
<i>longicarpus</i> ,* Roberts (1970)		1.7	2.4	2.9	3.4	2.4
<i>marshi</i> ,* Provenzano and Rice (1964)		2.3	2.7	3.2	4.0	—
<i>novae-zealandiae</i> ,* Greenwood (1966)		2.8	—	—	—	2.6
<i>pollicaris</i> ,* Nyblade (1970)		2.8	3.2	3.2	3.8	—
<i>prideauxi</i> , Goldstein and Bookhout (1972)		2.9	3.4	4.3	4.9	3.8
<i>prideauxi</i> , MacDonald <i>et al.</i> (1957)		3.4	4.2	5.2	6.0	4.3
<i>pubescens</i> , MacDonald <i>et al.</i> (1957)		3.1	3.9	4.6	5.4	3.1
<i>samuelis</i> ,* MacMillan (1971)		2.4	2.6	2.6	3.4	3.4
<i>samuelis</i> ,* Coffin (1960)		2.1	2.6	3.2	3.5	—
<i>sculptimanus</i> , MacDonald <i>et al.</i> (1957)		3.2	—	—	—	—
<i>variabilis</i> , Samuelson (1972)		5.2	6.0	6.4	—	—
Mean overall		2.7	3.3	4.0	4.2	2.8
Standard deviation overall		0.75	0.96	1.23	1.58	0.75
Mean starred species		2.3	2.7	3.1	3.7	2.5
S.D. starred species		0.33	0.32	0.25	0.25	0.57

* Twelve smaller species showing similarity.

In the photoperiod study the duration of stages at the 24:0 light treatment was 33 days (median), a variation of 2.5 days from the descriptive study median (35.5 days). Here culture conditions and collecting areas were similar. Based on available studies, it appears that larval stage duration is not a valid characteristic for species groupings, without considering optimal conditions for culture. For example, the three species described by Hall (unpublished, 1972), *P. hirsutiusculus*, *P. hemphilli*, and *P. granosinanus*, as well as *P. samuelis* from a California locality (MacMillan, 1971), show a greater similarity in stage duration than the two studies of *P. hirsutiusculus* collected from different localities and cultured at different temperatures.

Total length of individual zoea varied from the California study by 0.1 mm in Zoea II and Zoea III to 0.5 mm in Zoea I, Zoea IV, and the glaucothoe. All were larger in the Washington study. Culture conditions should not account for this difference. Zoea anatomical structure, judging from a comparison of the two studies' descriptions, appeared to vary little. Chromatophore patterns differed, possibly due to photoperiod variation (California 12:12 light vs. 24:0 Washington). Because of the similarities in anatomy, the observed differences in size and stage duration may indicate cultural variation and not taxonomic differences.

The number of eggs hatched per female had a close positive correlation with the size of the female (Table I). This appears confirmed by other studies of hermit crab larvae: the larger the female, the more eggs produced (Fotheringham, 1976). The negative correlation of hatch number with time of hatching in hours (Table I) may be due to the larger females being more efficient in effecting release of zoea from the egg mass via use of the antennae. Thus, hatching time decreases with increased egg numbers and increased female size.

Photoperiod length during laboratory culture does not appear to be an important condition in regulating zoeal growth or survival of this species (Table II). An investigation of larvae of the grapsid crab *Sesarma reticulatum* by Costlow and Bookhout (1962) also indicated no significant effects of varying photoperiods on mortality or stage duration.

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SUMMARY

Using laboratory cultured larvae of *P. hirsutiusculus* from Puget Sound, Washington, the durations of the four zoeal stages and the external anatomy and dissected appendages of the five larval stages are described and drawn. The species is placed within Group A of the established groupings of *Pagurus* larvae. Comparisons with a California study of the species show variation in stage duration

and body length, but minor differences in anatomy and appendages. A compilation of total larval body lengths from 19 *Pagurus* species is presented. Photo-period during larval development does not appear to be significant in regulating growth and survival of *P. hirsutiusculus*.

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PROTEIN SYNTHESIS IN LIVER, MUSCLE AND GILL OF MULLET (*MUGIL CEPHALUS* L.) IN VIVO¹

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The striped mullet is abundant in estuaries of the warm-temperate and subtropical regions of the world. This large herbivorous fish is an important species in aquaculture in the Pacific, Southeast Asia and the Mediterranean (Bardach, Ryther and McLarney, 1972). The species tolerates a wide range of salinities and temperatures (Moore, 1974). Optimal growth conditions are in the range of 20 ppt salinity (Vallet, Berhaut, Leray, Bonnet and Pic, 1970) and 20 to 24° C (Nash and Kuo, 1975). Mullet show seasonal acclimatization of metabolism typical of eurythermal fish (Moore, 1976) and a changing enzyme pattern with acclimation in the laboratory (Hochachka and Clayton-Hochachka, 1973).

The present study was directed toward evaluation of methods for quantitative measurement of protein synthesis in the mullet. Protein metabolism is recognized to play a major role in the growth and development of mammals (Munro, 1970). Recent studies have drawn attention to the importance of accurate determination of protein synthetic rates for improvement of meat production in domestic animals (Nicholas, Lobley and Harris, 1977). Such measurements could provide a criterion for assessment of growth potential under various conditions. This would be particularly valuable in fish culture, which is subject to such a large number of environmental variables.

Previous studies have provided evidence of high protein synthetic activity in fish. Polypeptide chain assembly rates in toadfish liver are comparable to those in mammals when temperature difference is taken into account (Haschemeyer, 1969; Nielsen, Plant and Haschemeyer, 1977). A quantitative study in goldfish brain *in vivo* indicated a synthetic rate at 34° C close to that observed in rat brain (Lajtha and Sershen, 1976). Liver and gill show similar synthetic rates, based on simple incorporation measurements, whereas muscle is one or two orders of magnitude lower (Das and Prosser, 1967; Smith and Haschemeyer, 1974; Narayansingh and Eales, 1975).

A variety of methods have become available in recent years for quantitative determination of protein synthetic rates *in vivo* (see review by Haschemeyer, 1976). Methods based on pulse injection of radioisotopes have been effective in liver of mammals (Mathews, Oronsky and Haschemeyer, 1973; Scornik, 1974) and fish (Mathews and Haschemeyer, 1978). Total body protein synthesis and individual tissues may be examined by the method of constant infusion (Garlick, Millward and James, 1973). These methods have now been applied to determine protein synthetic rates in tissues of mullet of the southeastern United States.

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MATERIALS AND METHODS

Adult striped mullet (*Mugil cephalus* L.), 200 to 300 g, were obtained with cast nets and seines in estuaries near the Belle W. Baruch Institute for Marine Biology and Coastal Research, Georgetown, S. C. Fish were maintained with aerated running seawater in circular tanks (5 ft diameter) at the ambient temperature of 28° C. Experiments were performed within one to five days after collection. Fish kept over 2 days were fed Clark's pellets. All fish were well-fed, as indicated by intestinal contents at autopsy.

Pulse injection experiments

For measurements of fractional incorporation rates and of polypeptide chain assembly time in liver, mullet were anesthetized with 0.05 g/l benzocaine (ethyl-p-aminobenzoate) and maintained with aerated seawater passing over the gills. The body cavity was opened and an injection of 2 μ Ci of L-[U-¹⁴C]leucine at a leucine concentration of 0.1 mM and [³H(G)]inulin in 0.1 ml buffered saline was made into an intestinal vein which joined the hepatic portal circulation. Following a labelling period of 2–4 min, the liver was rapidly excised, homogenized and fractionated to determine incorporation into *S* (soluble completed proteins) and *T* (total protein including ribosome-bound nascent chains), as described by Mathews, Oronsky and Haschemeyer (1973). Average polypeptide chain assembly time was obtained from the slope of *S/T* vs. time (Haschemeyer, 1969). Free radioactivity for the two isotopes was determined in the acid-soluble fraction of liver and of plasma collected from the region of the hepatic vein (Persell and Haschemeyer, 1976). Samples of the liver homogenates were analyzed for nucleic acids and protein, as described by Haschemeyer (1968).

The fraction of radioactive leucine incorporated into protein was obtained from the ratio of protein-bound ¹⁴C in μ Ci/g divided by total ¹⁴C radioactivity (protein-bound plus free) recovered in liver. The result for each fish was divided by incubation time (including 0.5 min for injection and work-up) to obtain fractional incorporation rate. This analysis assumes a relatively constant tissue-specific radioactivity and linear incorporation during the short incubation period. When combined with amino acid concentration in the tissue, the data provide an estimate of protein synthetic rate (Mathews and Haschemeyer, 1978).

Constant infusion experiments

Fish were anesthetized in seawater containing 0.1 g/l benzocaine and maintained on a surgical rack with circulating seawater. Cannulation of the dorsal aorta was carried out by the method of Soivio, Nynolm, and Westman (1975). In several fish a cannula was also inserted into the caudal vein to allow for serial blood sampling during infusion. After a recovery period of at least 4 hr, 7 μ Ci of L-[¹⁴C (U)]tyrosine (New England Nuclear) in 0.4 ml buffered saline was infused at a constant rate over a period of 4 hr. The fish were free-swimming in a small aquarium throughout this time. Temperature was 25 $\pm$ 1° C. In two experiments 5 μ Ci of ³H-inulin was included in the infusion solution to examine distribution in extracellular space. At the end of the infusion, caudal blood

samples were taken from all fish. Liver, gills, and a sample of white epaxial muscle were excised and homogenized by means of a Sorvall Omnimixer in 2 vol (liver, gills) or 4 vol (muscle) of Medium A (0.25 M sucrose, 50 mM Tris-HCl, pH 7.4; 25 mM KCl, 10 mM $MgCl_2$).

Incorporation into protein was determined for plasma and for the liver and gill homogenates on filter paper discs; free radioactivity in the trichloroacetic acid (TCA) supernatant was measured in Biofluor (Haschemeyer, 1969). Samples of tissue protein from 0.6 to 0.7 g tissue were obtained by TCA precipitation and washed with 5% TCA, 3% perchloric acid ($3\times$), absolute ethanol ($2\times$) and anhydrous ether ($2\times$). The final precipitates were dispersed into powder, thoroughly oven-dried and weighed. Ten milligram samples of muscle protein were incubated overnight in 0.5 ml Protosol (New England Nuclear) at 55° C, further incubated 2 hr with 0.3 ml 30% H_2O_2 for decolorization, and counted in 10 ml Biofluor after acidification with 0.5 ml of 0.5 N HCl. Fifty milligram samples of liver, gill, and muscle protein were hydrolyzed in 4 ml of constant boiling 6 N HCl in evacuated tubes at 110° C for 18 hr.

Specific radioactivity of L-tyrosine in the protein hydrolyzates and in the tissue acid-soluble free pools was determined after enzymatic conversion to tyramine and extraction as described by Garlick and Marshall (1972). Protein hydrolyzates in 3 ml water or 2 to 3 ml samples of the tissue TCA supernatants after ether extraction were incubated 1 hr at 37° C with 0.2 unit of L-tyrosine decarboxylase (Worthington) in 0.17 M citrate buffer, pH 5.5. Radioactivity in the final sulfuric acid extract was determined by counting in 5 ml Biofluor; tyramine was assayed by the nitrosonaphthol fluorometric method of Waalkes and Udenfriend (1957) and corrected for background due to enzyme alone.

Amino acid composition was determined in tissue protein samples to which norleucine was added as an internal standard at 10% by weight. Hydrolysis was carried out as above. Free pool concentrations were determined in aliquots of homogenate containing norleucine (500 nmol/g) and precipitated with 5% sulfosalicylic acid immediately after homogenization of the tissue. Rapidity of preparation was essential to avoid accumulation of free amino acids through protein degradation. Analyses were performed on a Technicon TSM Automatic Analyzer; calculations were based on norleucine content and machine response to a standard amino acid mixture (Pierce Chemicals).

Values of S_B (specific radioactivity of protein-bound amino acid) and S_f (specific radioactivity of free amino acid) were determined directly by tyramine analysis as described above. Apparent protein-bound specific radioactivity (S_B^{App}) was determined for each sample by use of the total incorporation into protein (from analysis on filter discs or in Protosol) and the protein-bound tyrosine content of each tissue. Apparent specific radioactivities were also calculated for the free pools (S_f^{App}) using total free radioactivity and tyrosine concentrations obtained by automatic analysis.

Values of the synthetic rate constant k_s (fraction of tissue protein replaced per day, including any growth increment) were determined from the equations of Garlick, Millward and James (1973). Results for liver and gill were based on the

TABLE I

Parameters for liver protein synthesis in pulse injection experiments; $T = 25^{\circ}\text{C}$, with standard deviation ($N = 5$).

^3H -inulin recovery (% of dose)	4.7 $\pm$ 0.6
^{14}C -leucine recovery (% of dose)	17 $\pm$ 5
Normalized plasma ratio ($^{14}\text{C}/^3\text{H}$)	0.40 $\pm$ 0.10
Fractional incorporation rate (min^{-1})	0.046 $\pm$ 0.025
Polypeptide chain assembly time (min)	3.0 $\pm$ 1.0
Liver ribonucleic acid (mg/g wet wt)	5.9 $\pm$ 1.0

following equation for tissues with rapid protein turnover:

$$S_B, S_i = \frac{\lambda_p}{\lambda_p - k_s} \cdot \frac{[1 - \exp(-k_s t)]}{[1 - \exp(-\lambda_p t)]} - \frac{k_s}{\lambda_p - k_s} \quad (1)$$

where λ_p is the time constant for exponential rise of plasma free radioactivity. Protein synthetic rate for muscle was obtained from:

$$S_B/S_i = \frac{R}{R - 1} \cdot \frac{[1 - \exp(-k_s t)]}{[1 - \exp(-Rk_s t)]} - \frac{1}{R - 1} \quad (2)$$

where R is the ratio of tyrosine in protein-bound form to that in the free pool. The calculation of k_s is not very sensitive to R , therefore a value of $R = 200$, based on the average free pool concentration, was used for all individuals.

RESULTS

Fractional incorporation rate in liver

Pulse injection of ^{14}C -leucine and ^3H -inulin into the hepatic portal circulation of mullet leads to rapid concentration of the amino acid in liver relative to the extracellular space marker. At times of 2 to 4 min after injection recovery of leucine averaged 17% of dose, compared to 4.7% for inulin (Table I). Plasma obtained after excision of the liver showed a normalized ^{14}C , ^3H ratio of about 0.4 compared with an initial value of 1.0 in the injection solution. This result indicates a high level of tissue uptake of the amino acid, similar to that studied in more detail in toadfish (Persell and Haschemeyer, 1976). The fractional rate of incorporation of leucine into protein, determined from the ratio of protein-bound to total ^{14}C radioactivity in liver, was $0.046 \pm 0.025 \text{ min}^{-1}$, or about 5%/minute. Individual variation is large because of differences in endogenous leucine pools, which determine effective specific radioactivity.

Because the pulse injection method does not yield a homogeneous distribution of the radioactive amino acid in liver (Persell and Haschemeyer, 1976), specific radioactivity determined for the tissue as a whole cannot be used to calculate protein synthetic rate. Fractional incorporation rate, however, provides an estimate of the rate of leucine pool utilization in cells that have taken up the isotope (Mathews and Haschemeyer, 1978). This result can be combined with free leucine concentration in the tissue to determine the amount of amino acid incorporated into

TABLE II

Concentrations of free amino acids in tissues of mullet. Means for acidic and neutral amino acids are presented $\pm$ standard deviation ($N = 11$).

	$\mu\text{mol/g wet weight}$		
	Liver	Gill	Muscle
Ala	3.0 $\pm$ 1.9	1.0 $\pm$ 0.5	4.2 $\pm$ 1.7
Asp	0.28 $\pm$ 0.12	0.40 $\pm$ 0.14	0.55 $\pm$ 0.24
Cys	0.15 $\pm$ 0.10	0.33 $\pm$ 0.22	0.67 $\pm$ 0.09
Gly	2.9 $\pm$ 0.6	1.5 $\pm$ 0.9	6.9 $\pm$ 1.4
Glu	5.0 $\pm$ 1.9	1.9 $\pm$ 1.5	0.55 $\pm$ 0.09
Ile	0.28 $\pm$ 0.08	0.25 $\pm$ 0.09	0.56 $\pm$ 0.10
Leu	0.50 $\pm$ 0.15	0.45 $\pm$ 0.14	0.83 $\pm$ 0.14
Met	0.14 $\pm$ 0.06	0.12 $\pm$ 0.06	0.19 $\pm$ 0.05
Phe	0.09 $\pm$ 0.03	0.12 $\pm$ 0.05	0.22 $\pm$ 0.06
Pro	0.40 $\pm$ 0.14	0.40 $\pm$ 0.20	0.54 $\pm$ 0.33
Ser	0.46 $\pm$ 0.22	0.44 $\pm$ 0.19	1.2 $\pm$ 0.3
Thr	0.24 $\pm$ 0.16	0.47 $\pm$ 0.30	1.0 $\pm$ 0.3
Tyr	0.05 $\pm$ 0.02	0.11 $\pm$ 0.03	0.21 $\pm$ 0.06
Val	0.18 $\pm$ 0.10	0.25 $\pm$ 0.12	0.11 $\pm$ 0.02

protein per unit time. The endogenous pool is assumed to be at the same concentration throughout the liver. On the basis of free leucine concentration in mullet liver (Table II) this calculation yields a leucine incorporation rate of $0.023 \mu\text{mol}/(\text{g} \cdot \text{min})$. Leucine content of mullet liver protein is $0.87 \mu\text{mol}/\text{mg}$ (Table III), or about 10% by weight. Combination of these values yields a protein synthetic rate of $26 \mu\text{g}/(\text{g} \cdot \text{min})$ or $37 \text{ mg}/(\text{g} \cdot \text{day})$. If 75% of synthesis is directed to intrahepatic protein, as indicated below, fractional daily synthesis (k_s) is given by:

$$\frac{(0.75)(37 \text{ mg}/(\text{g} \cdot \text{day}))}{140 \text{ mg/g}} = 0.20 \quad (3)$$

where the denominator is the protein concentration of mullet liver (Table III).

Polypeptide elongation rate in liver

Radioactivity in completed proteins relative to total incorporation into protein (including nascent chains) was plotted vs. time to obtain average polypeptide

TABLE III

Leucine and tyrosine content of mullet protein.

Tissue	Protein content (mg/g wet weight)	$\mu\text{mol leucine}$ (mg protein)	$\mu\text{mol tyrosine}$ (mg protein)	$\mu\text{mol bound tyrosine}$ (g tissue)
Liver	140 $\pm$ 10 (9)	0.87	0.17	24
Gill	90 $\pm$ 5 (6)	0.77	0.17	15
Muscle	190 $\pm$ 15 (8)	0.81	0.175	33

chain assembly time (Table I). The result, 3.0 min, represents an elongation rate of 2.2 amino acid residues/second, based on an average chain length of 390 residues (45,000 daltons), as in toadfish liver (Mathews and Haschemeyer, 1978). Ribosome concentration in mullet liver is about 1.75 nmol/g, based on liver RNA (Table I), with 80% taken to be ribosomal RNA, as in rat liver (Blobel and Potter, 1967). If all ribosomes are active, protein synthesis obtained from elongation rate is found to be 26 $\mu\text{g}/(\text{g}\cdot\text{min})$. The agreement between this result and that obtained from fractional incorporation rate above indicates that, within experimental error, the mullet liver is functioning at an optimal level.

Distribution of radioactivity during constant infusion

Plasma-free radioactivity in mullet showed an exponential rise to a plateau level, as in other species subjected to tyrosine infusion (Garlick and Marshall, 1972). The time constant λ_p was 25/day. Recoveries of free radioactivity in the various tissues relative to final plasma concentration after 4 hr infusion are given in Table IV. Levels for ^3H -inulin are consistent with values for extracellular space in fish (Holmes and Donaldson, 1969). Carbon-14 radioactivity was found to be concentrated in liver relative to plasma, whereas levels in gill were comparable to water space. Recovery in muscle was highly variable and may reflect incomplete distribution of isotope in some cases. This could be due in part to the use of a shorter infusion period than is typically used in mammals (Garlick, Millward, and James, 1973).

Tissue weights and total recoveries of carbon-14 in protein-bound and free form are summarized in Table V. Liver protein synthesis, based on the sum of results for liver and plasma, accounts for about two-thirds of measured incorporation into protein. The ratio of incorporation into plasma protein relative to total liver-plus-plasma protein was determined for each individual and averaged to yield 0.24 ± 0.03 (11). The result is consistent with studies in rats (Peters and Peters, 1972) and toadfish (Haschemeyer, 1973) that have shown about 20 to 25% of liver protein synthesis to be directed toward plasma proteins.

Fractional synthesis rates by constant infusion

Table VI summarizes incorporation data in fish analyzed for specific radioactivities by the tyramine procedure. Values of S_i in muscle were identical to S_i^{APP} calculated from total free radioactivity and tissue tyrosine content. This

TABLE IV

Tissue/plasma ratios of free (acid-soluble) radioactivity ($\pm$ standard deviation) following 4 hr of infusion of ^{14}C -tyrosine and ^3H -inulin in mullet at 25° C.

Isotope	(dpm/g tissue)/(dpm/ml plasma)		
	Liver	Gill	Muscle
^3H	0.26 ± 0.01 (2)	0.23 ± 0.01	0.15 ± 0.01
^{14}C	2.8 ± 1.0 (7)	0.83 ± 0.06	0.56 ± 0.31

TABLE V

Recovery of carbon-14 in mullet tissues following constant infusion of ^{14}C -tyrosine for 4 hr at 25° C (N = 11).

Tissue	Amount tissue (100 g body wt.)	% of Dose ($\pm$ SD)	
		Acid-soluble pool	Protein-bound
Liver	1.23 $\pm$ 0.19 g	1.7 $\pm$ 0.5	6.1 $\pm$ 2.5
Plasma	2.0 ml (estimated)	1.1 $\pm$ 0.3	1.9 $\pm$ 0.7
Gill	1.34 $\pm$ 0.20 g	0.6 $\pm$ 0.1	1.8 $\pm$ 0.5
Muscle	70 g (estimated)	20 $\pm$ 9	2.0 $\pm$ 0.8

indicates that no significant amount of acid-soluble carbon-14 was associated with substances other than L-tyrosine. In contrast, results in gill showed about 50% of radioactivity in other substances; in liver 75% of the free carbon-14 was not associated with L-tyrosine. Similar results obtained in rabbit liver have been attributed to transamination (Nicholas, Lobley and Harris, 1977). Oxidation also accounts for 10 to 20% of total tyrosine utilization in rats (Garlick, Burk and Swick, 1976). The high tissue/plasma ratio in mullet liver (Table IV) suggests an accumulation of metabolic products. Low levels of tyrosine in liver complicated the determination of S_i by tyramine analysis. Only two fish yielded reproducible data for S_i in liver.

Protein-bound specific radioactivities determined from incorporation levels and the tyrosine content of tissue protein (Table III) were identical to those obtained by tyramine analysis. Values of S_B used for calculation of (S_B/S_i) in Table VI represent averages from both methods of determination. The agreement between S_B and S_B^{APP} is an indication that radioactive tyrosine is not converted into other substances that may be incorporated into protein. A similar observation has been made in rabbits (Nicholas, Lobley and Harris, 1977).

Fractional synthesis rates in the various tissues were determined by means of equations (1) and (2). The value of $k_s = 0.20$ in liver, corresponding to 20% replacement of liver protein/day, is in agreement with the results obtained by pulse injection. This value refers to the synthesis of intrahepatic proteins only.

TABLE VI

Protein synthetic rates determined by tyramine analysis following constant infusion of ^{14}C -tyrosine in mullet; N = 7, except liver S_i (N = 2).

	Muscle	Gill	Liver
Free ^{14}C ($\mu\text{Ci/g}$)	0.008 $\pm$ 0.003	0.012 $\pm$ 0.004	0.044 $\pm$ 0.020
Protein-bound ^{14}C ($\mu\text{Ci/g}$)	0.0008 $\pm$ 0.0003	0.028 $\pm$ 0.013	0.12 $\pm$ 0.05
Free tyrosine ($\mu\text{mole/g}$)	0.17 $\pm$ 0.07	0.12 $\pm$ 0.02	0.042 $\pm$ 0.021
S_i^{APP} ($\mu\text{Ci}/\mu\text{mol}$)	0.050 $\pm$ 0.014	0.10 $\pm$ 0.05	1.0 $\pm$ 0.3
S_i ($\mu\text{Ci}/\mu\text{mol}$)	0.050 $\pm$ 0.021	0.065 $\pm$ 0.020	0.24 $\pm$ 0.05
(S_B/S_i) $\times 10^2$	0.45 $\pm$ 0.17	29 $\pm$ 7	25 $\pm$ 2
k_s (/day)	0.0054 $\pm$ 0.0020	0.23 $\pm$ 0.06	0.20 $\pm$ 0.02

Gill protein is replaced at a similarly high rate, whereas protein synthesis in white epaxial muscle at 0.5%/day is only 1/40 the rate in liver and gill.

DISCUSSION

The constant infusion technique has proven to be a powerful method for the study of protein metabolism in mammals (Waterlow and Garlick, 1975). During infusion a constant specific radioactivity is achieved in the free amino acid pools of the body, which is then used in the determination of synthetic rate. The present study establishes the feasibility of this technique in fish. Agreement in the results obtained by constant infusion and by pulse injection methods in liver suggests that the approximations inherent in the methods are sufficiently valid. Similar agreement has been obtained between constant infusion and other methods in studies of mammalian liver (Haschemeyer, 1976).

Liver protein synthesis in the mullet at 25° C is comparable to that observed in toadfish, a marine species of similar coastal habitat (Haschemeyer, 1969). The fractional synthetic rate (20%/day) in mullet may be compared with results in mammals by using the temperature dependency ($Q_{10} = 2.5$) determined for liver protein synthesis *in vivo* (Mathews and Haschemeyer, 1978). This calculation yields an equivalent value of k_s at 37° C of 60%/day. Values of 50 to 70% have been obtained by constant infusion in rat liver (Garlick, Millward, James and Waterlow, 1975).

The high rate of protein synthesis in gill is of interest in relation to the demands on this tissue for osmoregulation and nitrogen excretion. Inhibitors of protein synthesis have been found to cause pronounced effects on sodium exchange in gills of the eel (Maetz, Nibelle, Bornancin and Motais, 1969). The observed synthetic rate in gill, as in liver, can be given an equivalent 37° C value of about 60%/day, based on $Q_{10} = 2.5$. A value of 55%/day has been obtained in rat kidney (Garlick, Millward, James and Waterlow, 1975). Lung also falls in the category of tissues with rapid protein turnover (Garlick, Burk and Swick, 1976).

Mullet muscle shows a low rate of protein synthesis (about 0.5%/day) consistent with estimates based on incorporation data in other species, as cited earlier. The equivalent value of 1.5% at 37° C may be compared with values obtained in adults of various mammalian species: 4.9% in rat (Millward, Garlick, Stewart, Nnanyelugo and Waterlow, 1975); 4% in pig (Garlick, Burk and Swick, 1976); 1.9% in rabbit (Nicholas, Loble and Harris, 1977); and 1.8% in sheep (Buttery, Beckerton, Mitchell, Davies and Annison, 1975). Values of 1.1 to 2.4% have been obtained for myofibrillar protein synthesis in healthy human males (Halliday and McKeran, 1975).

Muscle protein synthetic rates in mammals show a close correlation with growth rate (Millward, Garlick, Stewart, Nnanyelugo and Waterlow, 1975) and diet (Garlick, Millward, James and Waterlow, 1975). Thus, this parameter is likely to be more useful than rates in liver and gill for assessing potential growth in fish. If no degradation of protein occurs in mullet muscle, the observed synthetic rate would represent a growth rate of 0.5%/day at 25° C. This optimal value, however, may never be achieved. In rat and pig only 20–25% of muscle protein synthesized each day is retained (Millward *et al.*, 1975; Garlick, Burk and Swick,

1976). Studies in rainbow trout, however, suggest that muscle protein retention is significantly greater in fish (Smith, in preparation).

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SUMMARY

1. Protein synthesis in mullet was measured by use of pulse injections of ^{14}C -leucine into the hepatic portal circulation and by 4-hr continuous infusion of ^{14}C -tyrosine into the dorsal aorta.

2. Liver protein synthesis was 37 mg/day per g wet weight of tissue. Plasma proteins accounted for about 25% of total liver protein synthesis. Replacement rate for intrahepatic proteins was 20%/day. Average polypeptide chain synthesis time was 3 min.

3. Gill protein synthesis was comparable to that in liver with a replacement rate of 23%/day.

4. Muscle protein synthetic rate was 0.54%/day, comparable to rates in rabbit and sheep when temperature difference is taken into account.

5. Constant infusion is shown to be a feasible technique for measurement of protein metabolism of fish *in vivo*.

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THE *LIMULUS* BIOASSAY FOR ECDYSTEROIDS¹

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Bioassays for molting hormone activity in arthropods have been used since Fraenkel's discovery of a tanning effect in ligated abdomens of the fly *Calliphora erythrocephala* after injection of hemolymph from pupariating larvae (Fraenkel, 1935). Whole animal extractions of numerous arthropods have been assayed and quantified for ecdysteroid content through the use of only a few test species. The two insect species *C. erythrocephala* (Fraenkel and Zdarek, 1970) and *Musca domestica* (Kaplanis, Tabor, Thompson, Robbins and Shortino, 1966) have been most widely used as bioassay organisms. The *Musca* test is the most sensitive and has been used to quantify crustacean (Willig and Keller, 1973) as well as insect extracts.

In recent years the techniques of radioimmune assay (Borst and O'Connor, 1972; Horn, Wilkie, Sage and O'Connor, 1976) and gas-liquid chromatography (Borst and O'Connor, 1974; Poole, Morgan and Bebbington, 1975), both sensitive assays, have tended to displace bioassays as routine detectors of ecdysteroids. With gas-liquid chromatography (GLC) as little as 10 to 20 pg can, after derivatization, be separated and identified (Ikekawa, Hattori, Rubio-Lightbourn, Miyazaki, Ishibashi and Mori, 1972; Borst and O'Connor, 1974). A good radioimmune assay (RIA), using an ³H-ecdysteroid of high specific activity, can detect about 100 pg of certain ecdysteroids (Borst and O'Connor, 1974). Both of these techniques require agents or equipment that may not readily be obtained or put to use by many workers who wish to study ecdysteroid levels or fluxes in arthropods. For them, a bioassay may be the most practical and efficient method of detection. According to Borst and O'Connor (1974), GLC and the *Musca* bioassay require further purification than RIA, except, at least, for hemolymph. In our experience, the reverse is true for RIA and the *Limulus* bioassay described in this paper. Winget and Herman (1976) found that extracts of juvenile *Limulus* were toxic to fly larvae in the *Musca* test, but they were able to assay extracts using the *Limulus* test. In short, we believe that there will be continued need for a good bioassay, preferably a non-insect one for marine arthropods, and therefore we present here some of our extensive work using first stage *Limulus* larvae as a test for ecdysteroid activity.

MATERIALS AND METHODS

Experimental animals

Only first stage *Limulus* larvae are suitable for the assay. Most of the work was performed on material collected on the Shackleford Banks near Beaufort, North

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Carolina. Material collected near Panama, Florida is equally suitable, and there is no reason to believe that geographical location of the collected material will have an effect on its usefulness as a bioassay. Larvae used in this study were collected in all stages of embryonic development and as freshly laid eggs. Eggs were obtained by marking the location of females when actually spawning, an event that most predictably occurs around high tides in the dark-time hours, and digging eggs out of the sand 4 to 5 hr later to ensure fertilization. Embryos were obtained by digging in the sand of the spawning beaches at the mid-tide mark. During June, clutches of developing embryos were usually discovered when digging for freshly laid eggs.

Freshly collected material was either taken to the Duke Marine Laboratory at Beaufort for work during the summer or transported to Gambier, Ohio for work during summer, fall and, occasionally, winter months. Eggs and embryos were cleaned from sand and debris within 12 hr of collection, placed in glass bowls, 8" diam (about 2000, bowl), containing filtered sea water, 30‰ salinity and transferred to environmental chambers. Natural sea water was used at Beaufort, artificial sea water at Gambier. At both locations eggs and embryos were stored at 25° C for use during the following 1 to 2 months and at 15° to 17° C for later use. Material from the low temperature was transferred to 25° C before hatched larvae were removed for aging and subsequent experimentation. Hatched larvae were routinely removed from the 8" culture bowls every 24 hr, and every 12 hr when critical timing was necessary, then transferred to 4½" diam glass bowls (about 200, bowl). During experimentation larvae of a specific age were tested in groups of 20, each group cultured in small glass bowls (3½" diam).

Steroids and reagents

Ecdysone was obtained from Dr. W. E. Scott and Hoffmann-LaRoche Chemical Co., 20-hydroxyecdysone and inokosterone from Rhotho Pharmaceuticals, Osaka, Japan. Cyasterone and poststerone were provided by Professor T. Takemoto, Tohoku University, Sendai, Japan; ponasterone A and C by Professor Koji Nakanishi, Columbia University, New York; 2-deoxy-20-hydroxyecdysone and podedysone A by Professors D. H. S. Horn and M. N. Gallbraith of CSIRO, Melbourne, Australia. Five synthetic analogs that lack hydroxyls in the side chain were gifts of Drs. William E. Robbins and Malcom J. Thompson of the USDA Insect Physiology Laboratory, Beltsville, Maryland. In this paper these analogs are designated as compound I (2 β , 3 β , 14 α -trihydroxy-5 β -cholest-7-en-6-one), compound II (2 β , 3 β , 5 β , 14 α -tetrahydroxy-5 β -cholest-7-en-6-one), compound III (2 β , 3 β , 14 α -trihydroxy-(24R)-5 β -ergost-7-en-6-one), compound IV (2 β , 3 β , 14 α -trihydroxy-27-nor-5 β -cholest-7-en-6-one) and compound V (2 β , 3 β -dihydroxy-5 β -cholest-7, 14-dien-6-one). Cholesterol and stigmaterol were purchased from Sigma Biochemical Co., St. Louis.

The natural ecdysteroids were dissolved in 20% ethanol or, occasionally, saline for injection. Synthetic ecdysteroid analogs were dissolved in 40 to 60% ethanol; sterols in 20 to 100% ethanol, depending on concentration of the sterol. Corresponding ethanol solutions were injected as solvent controls.

Bioassay technique

Tests were routinely conducted on first stage larvae at 25° C, in 30‰ seawater, and under a 13:11 hour light:dark regime. Twenty larvae were used for each test and control solution and uninjected controls, and incubated together in a 3½" diam bowl. Seawater was changed on alternate days during each experiment. For injection, a group of 20 larvae were selected at random from the larger holding bowls, partially dried on paper towels for about 1 min, then transferred to a deep-well depression slide for injection. All injections were performed under a Bausch & Lomb stereoscopic microscope at 7–10 × and were made through a glass pipette (15–20 μ tip diam) joined by polyethylene tubing (P.E. 20, Clay Adams) to a 10 μ l syringe (Hamilton, no. 701 plunger type, graduated to 0.1 μ l).

In practice, the pipettes were made from 1.0 mm O.D. glass tubing in a pipette puller, although they also can be pulled by hand in a gas flame. Pipettes with a total length of 80 to 100 mm and shank length of 10 to 15 mm were the most suitable. Polyethylene tubing was used in lengths of 30 to 35 cm, and each end was flared by a warm dissecting needle. One end could easily be attached to the needle of the syringe. The opposite end was attached to the glass pipette by heating the pipette in a gas flame and then gently working it into the flare of the tubing. Additional brief flaming of the glass and applied finger pressure to the glass and polyethylene tubings assured firm attachment. The assembled system of glass pipette, polyethylene tubing and syringe was filled with water or 20% ethanol—any air in the system could introduce errors in injection volume. A 1 to 5 ml syringe was used to fill the tubing and flush it after use. The tube of the syringe was filled by removing the plunger and introducing fluid through the plunger end; after inserting the plunger, fluid could then be drawn into the syringe without air bubbles. Attachment to the fluid-filled tubing provided a system free of air, and then 10 μ l of the test or control solution was drawn into the glass pipette. A small amount of mixing occurred at the interfaces of the two solutions; therefore, no more than 6 μ l were injected. This provided more than enough solution to successfully inject 20 animals and insured against injecting diluted test solution. The glass pipettes were large enough to confine the 10 μ l of experimental solution and avoid contamination of the polyethylene tubing. Then only the pipette alone required changing for a new kind of solution. Alternatively, a separate pipette-tubing system was used for each different kind of experimental solution.

After the system was readied for injections, the syringe was mounted in plasticine above the depression slide on the microscope stage, which permitted simultaneous viewing of both syringe barrel and larvae in the well of the glass slide. A larva was held against the vertical edge of the well with watchmaker's forceps, whereupon the larva would flex the body, and the tip of the glass pipette was inserted through the membrane between the prosoma and opisthosoma into the dorsal hemocoel of the prosoma. The larva was then raised from the bottom of the well with one hand while the plunger was moved slowly and smoothly with the other, all the while under visual inspection. The motion of injected fluid in the hemocoel could be observed and at the same time the larva usually extended the prosoma and moved the legs. These observations were indicators of success-

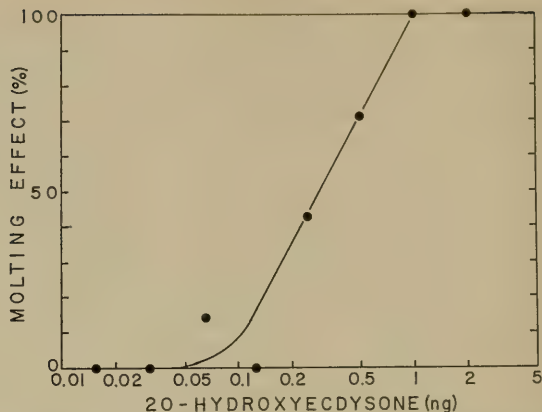


FIGURE 1. Response of first stage *Limulus* larvae to doses of 20-hydroxyecdysone in the range of 7.8 pg to 20 ng. The larvae were 15 to 16 days of age in a molt cycle of 23 days.

ful injections. Larvae were held on the pipette tip for about 20 sec after injection to insure entry of the entire injection volume into the hemocoel, then transferred to a labeled culture bowl. Larvae were discarded if they were injected with an excess of more than an estimated 5% of the experimental solution. Routinely, larvae were given one injection of 0.2 μ l of test solution (equals about 4% of the larval volume). Although they will tolerate 0.4 μ l, considerable swelling does occur with the larger volume.

The experiment was stopped 5 to 10 days after injection, depending on age of the larvae at time of injection. All animals were inspected for molting (actually throughout the experiment) or production of a new cuticle. The dorsal prosoma of unmolted larvae was dissected with watchmaker's forceps under the stereoscopic microscope and a new cuticle of about 1 μ m thickness was easily detected. Early apolysis was not reliably detected in gross dissection, therefore was not included as an effect. Animals molting before 96 hr post-injection time were discarded. Experimental effects were thus based solely on new cuticle formation and molting after a set period of time. The number of larvae affected among the survivors (usually 100%) was converted to a per cent effect; for experimental groups this was corrected for the corresponding control effect by the following method: if $100 - \% \text{ Control Effect} = Y$; and $100 - \% \text{ Experimental Effect} = X$; then $X \div Y = \% \text{ Effect, corrected}$.

Radioimmune assay technique

An antibody to 20-hydroxyecdysone was kindly provided by Professor G. R. Wyatt of Queens University, Kingston, Ontario, Canada. Antibody response to

TABLE I

Changing sensitivity of *Limulus* larvae to injected ecdysteroids during the 20-day molt cycle. Data were obtained from 1000 larvae.

Age after hatching (Days)	20-hydroxyecdysone		Ecdysone	
	Dose for 50% effect	Lowest detect- able dose	Dose for 50% effect	Lowest detect- able dose
2-3	54.0 ng	32.0 ng	63.0 ng	40.0 ng
4-5	34.0 ng	14.0 ng	56.0 ng	34.0 ng
6-7	8.4 ng	4.8 ng	8.4 ng	4.8 ng
8-9	4.3 ng	2.3 ng	3.8 ng	1.9 ng
10-11	460 pg	220 pg	530 pg	290 pg
12-13	90 pg	<20 pg	70 pg	<20 pg

ecdysteroids was assayed according to the method of Farr (1971) as modified by Borst and O'Connor (1972). ^3H -20-hydroxyecdysone at 3.3 c/mmole specific activity was purchased from New England Nuclear, Boston. The antiserum was diluted to bind 50 to 60% of the added radioactivity in control experiments that lacked unlabeled ecdysteroids, and rabbit serum was added to each assay tube to facilitate precipitation by ammonium sulphate. The final pellet was dissolved in water and counted for radioactivity in Triton-X scintillation fluid.

RESULTS

Sensitivity of the bioassay

Limulus larvae are very sensitive to injected ecdysteroids. Only 300 pg of 20-hydroxyecdysone accelerated molting in 50% of the larvae injected two-thirds through the molt cycle (Fig. 1). The dose-response curve is smooth from the lower limit of detection (about 200 pg in this batch) to a total effect. The *Limulus* bioassay is more sensitive than the *Musca* or *Calliphora* tests if the larvae, as a group, have aged to at least 50% through the molt cycle (Table I) or a time that corresponds to the beginning of premolt. First stage larvae from any one clutch of eggs progress towards their first post-hatch molt, but show a fair variation in the time of that molt. For example, the molt varied from 13 to 20 days for 20 larvae that averaged 18.0 days from hatch to molt, 20 to 27 for a batch that averaged 23.7 days. This variability is rarely a problem since the experimental larvae are measured against the control larvae, and the final values plotted for the standard curve are corrected for control values. At 25° C the first post-hatch molt cycle varies from about 15 to 25 days for batches of larvae hatched in the laboratory and maintained there during the summer months. It is shortest for larvae hatching at the time of collection and progressively longer for those with increasing time in culture conditions before hatching. Sensitivity of the larvae to exogenous ecdysteroids increases as larvae age from hatching to molting (Table I), and we have found that routine testing on 10- or 12-day-old larvae will provide a fairly sensitive test; in most cases more sensitive than the *Musca* or *Calliphora* tests.

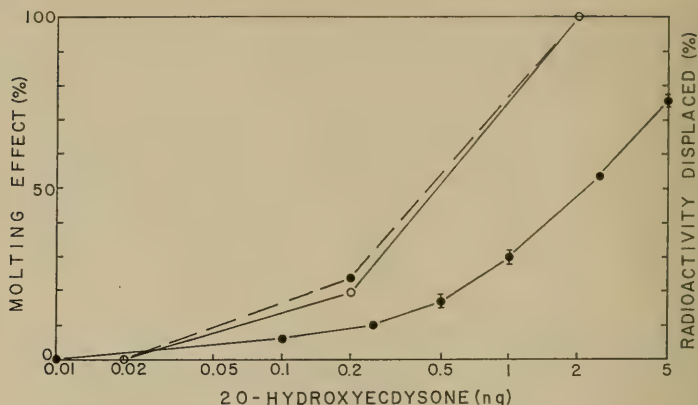


FIGURE 2. Comparison of the *Limulus* bioassay and a radioimmuno assay. For bioassay, 20-hydroxyecdysone was dissolved in saline (closed circles and broken lines) or 20% ethanol (open circles and solid lines) and injected into 10-day-old larvae on a 23-day molt cycle. RIA results (closed circles and solid lines) are plotted as the opposite of per cent bound; namely, per cent radioactivity displaced off antibody sites in the presence of measured amounts of ecdysteroid. The three points with vertical lines represent means of 17 to 20 experiments $\pm$ two standard errors of the mean; the others represent duplicate samples.

Data in Table I show a large (600- to 900-fold) spread in sensitivity of larvae of different ages to 20-hydroxyecdysone and ecdysone. Three doses were injected into groups of larvae of the youngest two ages, four in groups of the other ages. Animals used in these experiments were collected at the same time and all hatched within four days of each other; all experiments were terminated within three days of each other. These procedures ensured minimal physiological variation. The first significant sensitivity shift (four to sixfold) occurred in 6- to 7-day-old larvae, and coincides with a twofold increase in size of the epidermal cells; also, the cuticle is near its maximal intermolt thickness. A second increase in sensitivity occurred in 10- to 11-day-old larvae (seven to ninefold) and is associated with premolt events, based on data from histological work. A third sensitivity change occurred in 12- to 13-day-old larvae (five to sevenfold). At this time the new cuticle is just detectable in histological material. Larvae used after this time are not reliable for the bioassay, since there are many early molts that are not associated with the injected hormone. Whatever the length of the molt cycle, the bioassay seems to reach its ultimate sensitivity at the beginning of new cuticle formation. The limit of 20-hydroxyecdysone and ecdysone detection at that time is about 20 pg.

Usefulness of the bioassay

Limulus larvae seem unaffected by impurities in crude extracts of *Limulus* or by some strong alcoholic solutions. There were no apparent effects of 20, 50,

or 70% ethanol or methanol injections, although control larvae did not react well to 100% ethanol—the mortality varied from 25 to 35%. Stigmasterol or cholesterol in 100% ethanol, however, did not visibly have an adverse effect on the larvae; at least there was no significant mortality. Ecdysteroids were routinely dissolved in 20% (or stronger, if necessary) ethanol; but there is no significant difference in the results when 20-hydroxyecdysone is dissolved in saline or ethanol (Fig. 2). The control results for this experiment were identical.

A routine test on 10-day-old larvae detected lower amounts of 20-hydroxyecdysone than the RIA we used (Fig. 2). The RIA is, however, more selective in reaction to a variety of ecdysteroids. For example, cyasterone and makisterone A are potent stimulators of molting in the bioassay but have low activity in the RIA. Ecdysone, 20-hydroxyecdysone and ponasterone A are highly active in both systems. Since the *Limulus* bioassay is not selective, it can be used to quantify the total ecdysteroid content of an extract.

Developmental time and arrest for bioassay animals

Complete development from freshly laid egg to hatched larva can occur in 3 to 4 weeks at 25° C; it is, however, on the order of months at 15° to 17° C. Freshly laid eggs or embryos in early developmental stages that were collected in the summer and stored at 15° to 17° C remained suitable bioassay material for 6 to 9 months. We have discovered that larvae which have hatched buried in the sand or have been stored at below 20° C undergo a developmental arrest. Based on physiological data at 18° to 19° C, the arrest occurs after the first sensitivity change to injected ecdysones but definitely before premolt. The larvae may remain in this condition for several months, although some will eventually proceed to a molt at temperatures of about 17° to 20° C. Larvae crowded in the sand substrate will resume development upon transfer to ambient seawater, those at low temperature upon simple transfer to 25° C. A batch of eggs freshly laid in June and stored at 17° C had nearly all hatched 6 months later. These larvae developed rapidly and molted to the second stage seven days after transfer to 25° C. They were suitable for bioassay with a sensitivity of 100 pg of 20-hydroxyecdysone for a 50% effect. In another batch, collected as freshly laid eggs the end of July and stored in crowded conditions below 20° C, there were numerous unhatched larvae 5 to 6 months later. These were aged after hatching and used as bioassay material the following two months in a previously published study (Jegla, Costlow and Alspaugh, 1972).

Effectiveness of injected compounds

All of the natural ecdysteroids and one synthetic compound tested became increasingly effective as the molt cycle progressed from hatching (Table II). Cyasterone and the "triol", compound I, were far more effective early in the cycle than any other compound, and cyasterone was consistently the most effective ecdysteroid at all points in the molt cycle that we have tested. At the time of premolt cyasterone and ponasterone A were equally effective, with only about 50 pg necessary to cause a 50% molting effect. Ecdysone, 20-hydroxyecdysone, makisterone A, compounds I and II were of nearly equal potency and effective

TABLE II

Comparative effects of natural ecdysteroids, a possible ecdysteroid degradation product, synthetic ecdysteroids and sterols on *Limulus* larvae at three different points in the molt cycle (21-22 days for these experiments). Doses listed are for a 50% molting effect at 25° C. The compounds are listed in their estimated order of effectiveness, where an effect is shown.

Compound assayed	Time in the molt cycle		
	2-3 days	5-6 days	10-11 days
Cyasterone	2.3 ng	550 pg	49 pg
Ponasterone A	69.0 ng	1.05 ng	52 pg
Compound II	48.0 ng	820 pg	175 pg
Compound I	1.4 ng	5.2 ng	275 pg
Makisterone A	73.0 ng	4.3 ng	200 pg
20-hydroxyecdysone	66.0 ng	23.0 ng	200 pg
Ecdysone	87.0 ng	4.9 ng	230 pg
Ponasterone C	47.0 ng	5.7 ng	580 pg
Inokosterone	54.0 ng	34.0 ng	480 pg
Podecdysone A	71.0 ng	7.4 ng	1.4 ng
Compound IV	—	—	1.0 ng
2-deoxy-20-hydroxyecdysone	92.0 ng	56.0 ng	5.3 ng
Compound V	—	—	No effect
Compound III	No effect	No effect	No effect
Poststerone	No effect	No effect	No effect
Cholesterol	No effect	No effect	No effect
Stigmasterol	No effect	No effect	No effect

at about 200 pg in the 10- to 11-day-old larvae. Inokosterone and ponasterone C were less effective, followed by podecdysone A, compound IV, and 2-deoxy-20-hydroxyecdysone. The two synthetic compounds (III and IV), as well as poststerone, cholesterol and stigmasterol were ineffective.

DISCUSSION

The *Limulus* larva is a sensitive test organism for ecdysteroids. The most sensitive batch of larvae used in this study permitted detection of as little as 20 pg of 20-hydroxyecdysone, and this compares favorably with detection by the GLC method using an electron capture detector. Using the latter technique, Bebbington, Morgan and Poole (1977) were able to detect ecdysteroids in the low picogram range in barnacles. Since no one has yet determined reliable staging criteria for first stage *Limulus* larvae, the most sensitive animals can only be found by systematic testing throughout the latter half of the molt cycle. Accurate staging of the larvae should allow one to choose the most sensitive animals with a high degree of certainty. Routine testing on 10-day-old *Limulus* larvae will usually produce a bioassay with a sensitivity in the 300 to 500 pg range, and, in practice, this compares favorably with radioimmune assays.

On a per gram basis, *Limulus* larvae show a sensitivity to 20-hydroxyecdysone that is similar to barnacles (Tighe-Ford, 1977), crayfish (Warner and Stevenson, 1972), houseflies (Kaplanis *et al.*, 1966), flesh flies (Ohtaki, Milkman and Williams, 1967) and blowflies (Fraenkel and Zdarek, 1970). Specific tests are, how-

ever, conducted on individuals, where size alone plays a role in determining sensitivity of an organism. The *Musca* test is the most sensitive insect bioassay and houseflies are the smallest test organisms. *Limulus* larvae at a mass of 5 mg larva are about three times smaller than housefly abdomens and, on this basis alone, are more sensitive. Specificity in a physiological system must also have a role in the effect. Increased sensitivity of the *Musca* test compared to the *Calliphora* test cannot be accounted for by size alone (Kaplanis *et al.*, 1966). This also appears to be the case with *Limulus* compared to the various species of flies. The *Musca* and *Calliphora* assays enable detection of 20-hydroxyecdysone in the 2 to 10 ng range, whereas detection is an order of magnitude lower in the *Limulus* assay. Sensitivity is also clearly a function of time in the molt cycle. The fly bioassays are most sensitive very near the onset of pupariation (Karlson and Shaaya, 1964; Fraenkel and Zdarek, 1970). The *Limulus* bioassay as described in this paper is most sensitive at the beginning of new cuticle formation.

Structure-function studies of ecdysteroids and analogs on a number of arthropod systems have been performed in an effort to understand biosynthetic and degradative pathways of the molting hormones. The isolated *Musca* abdomen (Robbins, Kaplanis, Thompson, Shortino and Joyner, 1970), and *Calliphora* abdomen (Galbraith, Horn, Middleton, Thomson and Wilkie, 1975) are used most often in this kind of study. Effects on more specialized *in vitro* systems have also been used; for examples, imaginal wing disks of *Plodia interpunctella* (Oberlander, 1974) and cockroach leg regenerants (Marks, Robbins and Thompson, 1978). The system of Marks allows one to isolate the effects on and capabilities of specific organs. The present study utilizes a whole animal system, which allows one to assess what an animal, with all its biosynthetic and degradative pathways, as well as possible feedback mechanisms, can perform when exposed to ecdysteroids, their antagonists and inhibitors.

All ecdysteroids and analogs (except compound I) that were tested and proved effective in *Limulus* larvae at different times in the molt cycle increase in effectiveness as the larvae age from hatching. Only cyasterone and compound I are very effective in the "postmolt" period, 2 to 3 days after hatching (The larvae actually undergo their last prebatch molt shortly before hatching). Cyasterone with its lactone ring in the side chain is the most effective molt stimulator in *Limulus* and *Samia cynthia* pupae (Williams, 1968). Cyasterone's effectiveness may be due to its slow inactivation compared to ecdysone (Ohtaki and Williams, 1970). This is a reasonable hypothesis for its action in *Limulus* larvae based on the relative inactivities of ecdysone and 20-hydroxyecdysone soon after hatching. Compound I, which has the ecdysone nucleus but lacks hydroxyls on the side chain, generates ecdysone and 20-hydroxyecdysone by conversion in *Manduca sexta* (Kaplanis, Robbins, Thompson, and Baumhover, 1969) and *Calliphora stygia* (Thomson, Horn, Galbraith and Middleton, 1974). The higher activity of compound I would appear to exclude such a mechanism in *Limulus* larvae.

Structure-function studies on the *in vivo* insect systems are done on larvae of physiological age which corresponds to *Limulus* larvae in premolt. Therefore, for comparative purposes only, the data given in Table II for 10- to 11-day-old larvae are appropriate for use. The data indicate similarities as well as differences between *Limulus* and the insect systems. Cyasterone and ponasterone A are the

most effective ecdysteroids in premolt *Limulus*, and they are also the most effective in *Sarcophaga peregrina* (Ohtaki, Milkman and Williams, 1967). This is not the case in the *Musca*, *Calliphora* and *in vitro* insect systems. The ecdysone nucleus can be modified to some extent before the molt stimulating activity of a compound is interfered with. The 5- β and 14- α functions are necessary for activity in the *Musca* test (Robbins *et al.*, 1970) and in *Limulus* larvae. Compounds that have a 5- α -OH or lack the 14- α -OH are inactive in *Limulus* (Jegla *et al.*, 1972); compound V in this study lacks the 14-OH and has a 14-en function, but is also inactive. Compounds with a 5- β -OH are effective in both test systems (compound II and ponasterone C in this study). In the *Calliphora* test 2-deoxy-20-hydroxyecdysone is an effective agent but in *Limulus* larvae it is the least effective of the 12 compounds that stimulated molting. The side chain is also an important part of the molecule for molt stimulation, although side chain hydroxyls are not necessary on an injected compound. Poststerone, a 21-carbon compound with the ecdysone nucleus but without the cholestan side chain, is inactive in *Limulus* throughout the molt cycle. Compound III, which varies from I only in the addition of a C-24 methyl group, is also inactive throughout the molt cycle. Addition of the C-24 methyl group to ecdysone, as in makisterone A, does not interfere with molt stimulating activity. The compound 2-deoxy-20-hydroxyecdysone has been isolated from the crustacean *Jasus lalandei* and is highly active in the *Calliphora* test, suggesting, according to Thomson *et al.*, (1974), an efficient conversion to the molting hormone 20-hydroxyecdysone. The 2-deoxy ecdysteroid shows low activity in *Limulus* larvae, which does not suggest efficient conversion to molting hormone. Although 20-hydroxyecdysone is widespread in occurrence and may be a general arthropod molting hormone, the current evidence suggests specific differences in synthesis and conversion at the species, or higher, level.

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SUMMARY

This paper presents a description of the *Limulus* larval bioassay for ecdysteroid compounds. The *Limulus* assay is routinely more sensitive than the traditional insect tests and can detect 20-hydroxyecdysone in the low picogram range when test larvae are at the point of new cuticle formation. It compares favorably with detection of ecdysteroids by radioimmune assay for routine use. *Limulus* larvae can detect a wide range of natural ecdysteroids and synthetic analogs and can tolerate high concentrations of ethanol or methanol solutions. Therefore, it may be a useful assay for crude alcohol extracts and tests of fractions for total ecdysteroid content. Structure-function studies show similarities and differences between *Limulus* and some insect systems. Premolt larvae can detect cyasterone and ponasterone A in the low picogram range, 2-deoxy-20-hydroxyecdysone in the low nanogram range. The latter compound, which is very effective in *Calliphora stygia*, is the least effective of the natural ecdysteroids tested in *Limulus* larvae.

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A HISTOLOGICAL AND HISTOCHEMICAL STUDY OF DIGESTION IN THE BIVALVE *ARCTICA ISLANDICA* L.¹

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Yonge (1926a) considered the relative importance of extra- and intracellular digestion in the oyster *Ostrea edulis* and concluded that the bulk of digestion was intracellular, in amoebocytes or in cells of the digestive gland. Yonge felt that the only extracellular enzymes of consequence were carbohydrases from the crystalline style, and that the traces of proteases and lipases found in the stomach were derived from burst phagocytes. Since that time, however, there has been a growing appreciation of the importance of extracellular digestive processes in bivalves. George (1952) showed that extracellular lipolysis does occur in the stomachs of *Crassostrea virginica* and *Geukensia demissa*. Mansour (1946) found significant amounts of proteolytic and lipolytic enzymes in lamellibranch stomachs and hypothesized that these enzymes originated in spheres periodically pinched off and excreted from digestive gland cells. Morton (1956) found that preliminary breakdown of diatoms and flagellates occurred in the stomach of *Lasaca rubra*, a bivalve which lacks gut amoebocytes. Like Mansour, Morton concluded that digestion was probably mediated by free enzymes contained in excretory (= fragmentation) spheres. Reid (1968) constructed zymograms for extracellular esterases found in the gastric juices of twelve species of bivalves, including *Arctica islandica*. Recently, high activity of cathepsin B, trypsin, leucine aminopeptidase, and carboxypeptidase B have been demonstrated in the gastric juice of the bivalve *Tresus capax* (Reid and Rauchert, 1976). The importance of acid endopeptidases in preliminary extracellular digestion now seems well established for the bivalves.

While the role of the crystalline style in the secretion of amylases and other carbohydrases is well-documented (Owen, 1974), the source of extracellular esterases, lipases, and proteases in the bivalve stomach is far from clear. Excretory spheres from digestive cells remain a problematical source for extracellular enzymes, since in many species, they seem to be isolated from general stomach circulation by the disposition of the major typhlosole, and may appear intact in the feces (Owen, 1955, 1966, 1973). Histochemical surveys of the digestive tracts of *Ostrea edulis* and *Crassostrea angulata* indicate that the digestive ducts are active areas of enzyme secretion (Mathers, 1973). Ultrastructural studies, on the other hand, suggest that basophilic crypt cells in the digestive tubules elaborate a proteinaceous secretory product, likely including extracellular enzymes (Sumner, 1966b; McQuiston, 1969; Owen, 1970).

Establishing the source(s) of extracellular enzymes in lamellibranchs may have a bearing on a number of related questions. If enzymes are derived from fragmentation spheres, rhythmic production of these spheres (J. E. Morton, 1956; B. S.

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Morton, 1973) might account for some of the variability in extracellular enzyme activity observed in closely related species of bivalves (Reid and Rauchert, 1972; Reid, 1978). Alternatively, identification of specific enzymes in the apical granules of basophilic cells would contribute to our understanding of the function of various cell types in the bivalve digestive gland.

This study reports on the cytology of the digestive gland and the distribution of several hydrolytic enzymes in the stomach and digestive gland of the mahogany quahog *Arctica islandica* Linné. Source of extracellular enzymes and function of cell types in the digestive gland are also considered.

MATERIALS AND METHODS

Adult *Arctica islandica*, from 5 to 12 cm in length, were obtained from commercial fishing vessels operating off Point Judith, Rhode Island, between September, 1974 and March, 1975. Specimens were maintained at the Marine Science Institute, Nahant, Mass., in large tanks with a flow-through seawater supply.

Histology

Selected portions of the digestive system were fixed in Helly's, Hollande's or Zenker's fluid, embedded in polyester wax, and sectioned at 4 to 8 μm . For routine histological observations, sections were stained with Heidenhain's Azan or with hematoxylin and 0.5% Biebrich's scarlet. Alcian blue, pH 2.5, with Galigher's hemalum (Galigher and Kozloff, 1971) stained goblet cells. Iron hematoxylin or azocarmine following Helly's fixative was used to stain secretion granules (Sumner, 1966a). Toluidine blue (1%) in 1% borax and chlorazol black were used in demonstrating inclusions in digestive cells. PAS reagent and bromphenol blue were used for localization of polysaccharides and proteins, respectively. To test for insoluble deposits of calcium salts in basophil cells, neutral formalin-fixed sections were immersed in 10% cobalt nitrate, washed thoroughly, and treated with dilute ammonium sulfide (Gomori, 1952).

Enzyme histochemistry

Excised digestive gland, stomach wall, and anterior intestine from 25 quahogs were fixed at 4° C for 2 to 12 hr in 10% formalin, buffered to pH 7.0 with sodium phosphate buffer. The tissue was rinsed in cold distilled water, dehydrated in graded acetones, cleared in xylene, and embedded in 45° paraffin wax. Sections 10 to 15 μm thick were incubated according to one of the following histochemical techniques (incubation times indicated):

Exopeptidases: The Burstone and Folk (1956) L-leucyl- β -naphthylamide HCl method (1–1.5 hr). Originally intended as a specific method for leucine aminopeptidases, it has been shown that this substrate is attacked by a variety of peptidases, the great majority of which are exopeptidases (Gibson and Jennings, 1967).

Cathepsin C-type endopeptidases: The Hess and Pearse (1958) indoxyl acetate method, with 10^{-3} M cysteine hydrochloride as an activator and 10^{-3} M lead nitrate as an inhibitor.

Non-specific esterases: The Holt and Withers (1952) indoxyl acetate method (30–45 min).

Alkaline phosphatases: The Gomori (1952) calcium salt method (2–2.5 hr).

Acid phosphatases: The Burstone (1958) naphthol AS-BI phosphate method (3–4 hr).

Lipases: The Gomori (1952) Tween 80 method (2–48 hr). As a further test of lipolytic activity, minces of crystalline style or digestive gland were incubated according to the olive oil-Nile Blue Sulfate method of George (1952).

All of the histochemically treated sections were washed well in distilled water and mounted in glycerol jelly. Control incubations were of two types: sections were either heated to 90 °C for 5 min to denature enzymes, or the substrate was omitted from the incubation medium.

Feeding experiments

Colloidal graphite ("Aquadag", Fisher Scientific Co.) was suspended in seawater and pipetted into the mantle cavity of *Arctica islandica* to follow the transport and phagocytosis of small particulate material in the digestive tract. Animals were fixed at intervals following ingestion of graphite. Unstained sections proved most useful in locating the graphite particles.

Further experiments were designed to test the variability of enzyme production in the digestive tract. For these investigations, 15 *Arctica islandica* were starved for 2 to 4 weeks and then fed mixed cultures of the unicellular algae *Isochrysis galbana* and *Phaeodactylum tricornutum*. The concentration of algal cells was monitored regularly over an 8 hr period so that it remained at approximately 5000 cells/ml. After 1, 2, 4, 6, and 8 hr of feeding, individuals of *A. islandica* were fixed and prepared for histochemical examination.

RESULTS

This study centers on specific aspects of the cytology and histochemistry of the digestive system of *Arctica islandica*. Background information regarding anatomy of bivalve digestive organs may be found in: Reid (1965), who has described the ciliary patterns and structure of the stomach of *A. islandica*; Morton (1956) and Owen (1972), who have detailed the morphological variability of digestive cells in bivalves, and the relationship between this variability and feeding state; and Langton (1975), who has described the holding, absorptive, disintegrating, and reconstituting phases for the digestive cells of *Mytilus edulis*.

Histology

The stomach communicates with the digestive gland by a system of primary and secondary ducts. Most of the primary ducts (approximately 20) originate in either the left or the right duct cecum, which are recessed pockets of the stomach which open into the ventral portion of the main body of the stomach. Each of the primary ducts tapers to about 200 μ m in diameter as it passes from the stomach (one of the duct caeca) to the periphery of the digestive gland. At regular intervals, each primary duct branches into a cluster of 4 to 6 secondary ducts, which

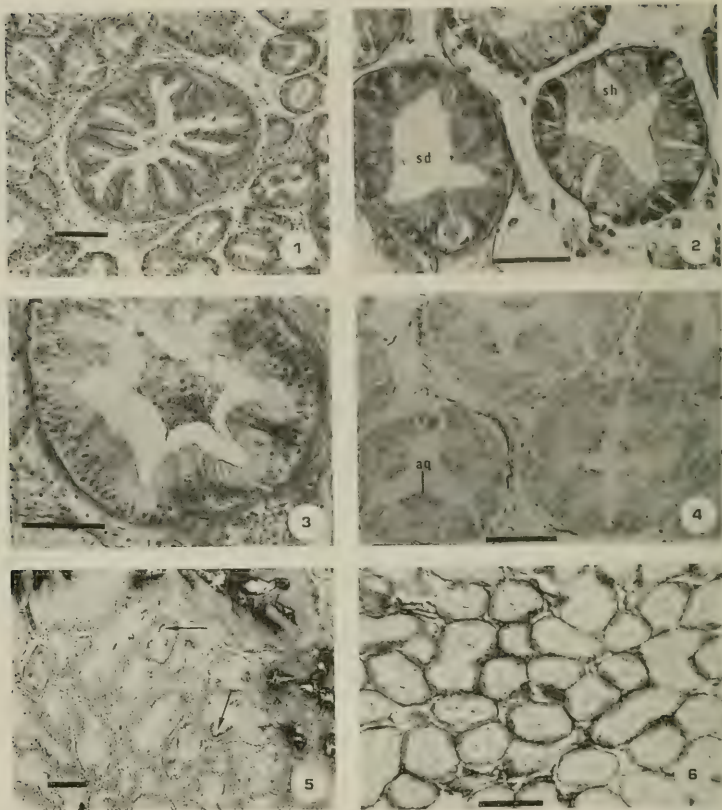


FIGURE 1. Primary duct near junction with duct cecum. Note the ridged, uniformly ciliated construction of the primary duct. (Azan; scale 100 μ m.)

FIGURE 2. Shedding of type A digestive cells. (Hematoxylin and Biebrich's scarlet; scale 50 μ m; sd, secondary duct; sh, shed digestive cell.)

FIGURE 3. Fragmentation spheres passing down lumen of primary duct toward duct cecum (stomach). Note nuclei of amoebocytes associated with the spheres. (Azan; scale 100 μ m.)

FIGURE 4. Aquadag collecting in distal inclusions of type A digestive cells. (Toluidine blue; scale 50 μ m; aq, aquadag.)

FIGURE 5. Alkaline phosphatase activity after 1 hr of feeding following starvation. Wall of duct cecum (left top), primary duct (right top), secondary ducts (right), and distal border of basophilic cells (arrows) are positive; digestive cells are negative. (Scale 150 μ m.)

FIGURE 6. Alkaline phosphatase activity after 6 hr of feeding following starvation. Basal portions of tubule cells are positive. (Scale 100 μ m.)

are short (80–100 μm) and narrow (60–70 μm). Each secondary duct then opens into a cluster of 3 to 5 digestive tubules, from 150 to 350 μm in length.

There is great histological variation from the duct ceca to the digestive tubules. The smooth epithelium of each duct cecum contains both ciliated columnar cells and alcianophilic goblet cells. With the transition from cecum to primary duct, the goblet cells disappear and the ciliated epithelium becomes folded. Each primary duct is organized into about 10 ridges and furrows, steep and sharply demarcated near the cecum and smoother in the body of the gland (Fig. 1). The primary ducts in *Arctica islandica*, then, are ridged and ciliated over the entire surface. In contrast, in the anisomyarian bivalves examined by Owen (1955), the epithelium of the primary ducts is separated into a well-defined ciliated tract and a brush-border epithelium. The epithelium of the ducts in *A. islandica* is also characterized by numerous amoebocyte-bound granules, up to 25 μm in diameter, which stain strongly with PAS and aniline blue.

The epithelium of the secondary ducts is low columnar to cuboidal, and the apical border of these cells shows a dense brush border, which stains vividly with Biebrich's scarlet. Like the primary duct cells, the secondary duct cells are slightly basophilic and show no obvious intracellular inclusions.

As in many other bivalves, the epithelium of the digestive tubules in *Arctica islandica* appears to contain two basic cell types: basophilic and digestive. In cross-sections of digestive tubules, basophilic cells are located at the crypts of the tubule lumen, while digestive cells are located on ridges between the tubule crypts. Both basophilic and digestive cells occur in two distinct types in *A. islandica*. Type A basophils are tall, tapering columnar cells, located centrally in the crypts, which stain less intensely with basic dyes than do the triangular type B basophils bordering the digestive cells. Further, in some sections, type A basophils show long flagella. Mitotic figures in the digestive tubules were only observed among type A basophils, and are visible in about 0.5% of all tubule cross-sections (6 μm sections). Although the nucleus in Type A basophils is normally a basally located, oval structure, it migrates prior to division so that granular mitotic figures are observed in the distal one-third of the cell. Type B basophils have a large basal nucleus, with a single, intensely staining nucleolus and fine-stranded chromatin. Mitotic figures were never observed in type B basophils. The cytoplasm of all basophil cells in *A. islandica* stains strongly and evenly with bromphenol blue, lightly with PAS, and negatively for calcium. There is no evidence of secretion granules in the cytoplasm, either with bromphenol blue or with iron hematoxylin or azocarmine following fixation with Helly's fluid.

Generally the digestive cell in *Arctica islandica* can be divided into three regions on the basis of the size and distribution of vacuolar inclusions. At the apical border there is a layer of unstained to lightly basophilic cytoplasm, which appears finely granular or dotted with fine inclusions. The acidophilic mid-cell region is packed densely with roughly spherical inclusions, up to 3 μm in diameter. The contents are pale yellow to pale green in unstained preparations from freshly collected animals, but tend to assume the color of any cytoplasmic stain. The supranuclear region of many digestive cells contains a single inclusion, as large as $17 \times 14 \mu\text{m}$. Other digestive cells, particularly those nearer the crypts, lack this large basal vacuole, and the supranuclear region is packed with the same inclusions that fill the mid-cell region. In fixed preparations, the contents of the

basal vacuoles consist of tightly packed, light yellow-green to yellow-brown inclusions, similar to those in the mid-cell region, which remain unstained after bromophenol blue or osmic acid. Unlike the inclusions in the primary and secondary duct epithelium, the basal vacuoles also do not stain with PAS; they are best visualized in longitudinal sections of the tubule stained with Biebrich's scarlet or chlorazol black.

Variability in the number and the staining properties of these inclusions allows distinctions to be made between neighboring digestive cells. The two or three digestive cells located centrally in a row of cells (Type A digestive cells) are often taller, bulge more conspicuously into the lumen of the tubule, and contain larger, more densely packed basal vacuoles than the digestive cells nearer the crypts (Type B digestive cells). The digestive cells also show a metachromasia with toluidine blue which allows their differentiation into two distinct types. Type B digestive cells show a uniform, pale violet, alcohol-fast β -metachromasia of both the spherical mid-cell inclusions and the background cytoplasm, while type A digestive cells contain small (0.5–2.0 μ m), irregular, deep violet β -metachromatic inclusions, located in the apical basophilic layer. This metachromatic reaction occurs consistently through the entire digestive gland.

Two other histological observations support the separation of digestive cells into two distinct types. First, the nuclei of type A digestive cells often show considerable variation. While some are similar to nuclei of type B digestive cells (large, oval and basally located, with a single spherical nucleolus, and very little visible chromatin in the nucleoplasm), many are highly condensed stellate structures which are compressed against the cell wall by the large basal vacuole. Under phase microscopy, compressed nuclei from type A digestive cells contain numerous, irregularly shaped, orange-red inclusions, similar in color to the nucleolus of a typical nucleus. In serial sections of some digestive tubules, nuclei of some type A digestive cells have degenerated and are not visible. Second, feeding studies show that phagocytosis of Aquadag particles is performed exclusively by type A digestive cells (See "Starvation and feeding experiments" and Fig. 6D in Owen, 1955).

The transition between basophilic and digestive cells is usually described as quite sharp in bivalves. This is normally the case in *Arctica islandica*, although there are significant exceptions. With toluidine blue, for example, there are numerous cases of cells apparently intermediate between basophils and digestive cells. The cytoplasm of these cells is strongly basophilic (orthochromatic), but contains inclusions characteristic of digestive cells. In Azan preparations, many cases have been observed in which the two basic cell types alternate over a length of three or even four cells along the basement membrane.

Fragmentation or excretion by digestive cells occurs in one of two ways in *Arctica islandica*. The distal portion of the cell, with numerous mid-cell inclusions, may be pinched off, or the entire cell may be shed (Fig. 2). Shedding is selective, affecting only type A digestive cells. In either case, when dense fragments of digestive cells occur in the tubule lumina, they are invariably surrounded by masses of amoebocytes (Fig. 3); at times when fragmentation is not apparent, the lumina are devoid of amoebocytes. Mid-cell inclusions are recognizable by

their size and staining properties in the ducts and duct ceca, but are not observed in the hindgut or in the feces.

Enzyme histochemistry

Exopeptidases. A bright red staining reaction occurred routinely in granules at the distal border of the primary and secondary duct cells. Also, in some sections the distal border of the ciliated cells of the duct ceca showed a weak positive reaction for exopeptidases. In the primary ducts, this reaction was more pronounced in the ciliated cells on the ridges than in the cells in the troughs; in the secondary ducts the bright red azo stain was evenly distributed along the luminal edge of the duct epithelium. Further, the portion of the primary duct nearest the stomach stained less intensely than either the distal portion of the primary duct or the secondary ducts. Exopeptidase activity abruptly stopped at the border of the secondary duct and digestive tubule, and none of the tubule cells ever showed activity.

Cathepsin C-like endopeptidases. Diffuse endopeptidase activity was normally evident in the cytoplasm of both type A and type B digestive cells. Optimum activity in these cells occurred near pH 5.2. The mid-cell region was most strongly colored by the oxidized indigo dye, while the basal vacuoles showed little or no reaction. Basophil cells, the primary and secondary ducts, and the ceca were negative for endopeptidases. A uniform blue reaction was consistently observed in luminal material derived from fragmenting digestive cells. These cell fragments, which could be seen in section in the ducts and duct ceca, showed an intensity of staining comparable to that observed in the digestive cells.

Non-specific esterases. Localization of non-specific esterases closely paralleled that of endopeptidases, with two exceptions. At pH 7.2 esterase activity was normally evident at the distal border of the primary duct epithelium. Although the intensity of the stain was weak and inconsistent, the proximal part of the duct showed the greatest activity, and the cells on the ridges were more active than those in the troughs. The epithelium of the duct ceca and stomach wall was also lightly positive for esterase activity, while the midgut was negative. As was the case with endopeptidase-like activity, fragmenting digestive cells contributed considerable esterase activity to the lumina of the stomach and the ducts.

Alkaline phosphatases. The distal border of all primary and secondary ducts showed a uniform, intense reaction following the calcium salt method (Fig. 5). The epithelium of the duct ceca and stomach wall also showed alkaline phosphatase activity, although the stain was much weaker in this region than in the ducts. The black precipitate abruptly stopped at the border of the secondary duct and digestive tubule; basophil and digestive cells were normally either negative or, in localized areas, weakly positive. The alkaline phosphatase activity of the basophil cells, when present, was located distally, while the digestive cells showed a thin band of activity either distally or along the basement membrane. Specific localization of alkaline phosphatase during a feeding cycle is described below.

Acid phosphatases. The distal border of the primary and secondary ducts and the epithelium of the duct ceca showed a weak to moderate reaction for acid

phosphatase. Basophil cells were consistently negative for acid phosphatase activity, while the digestive cells reacted variably. In many preparations, type A digestive cells showed a diffuse positive reaction in the distal two-thirds of the cell with the naphthol phosphate method. It was not possible to ascribe acid phosphatase activity to specific vacuoles; however, it is clear that the distal basophilic layer, the nucleus, and the basal vacuole were negative, while the region of the cell containing the densely packed, mid-cell inclusions was positive.

Lipases. No lipase activity was detectable in the stomach wall, digestive gland, or midgut with the Tween 80 method. Lipase activity was detected in a mince of the crystalline style, but not of the digestive gland, by use of the Nile Blue Sulfate method (12 hr incubation).

Feeding and starvation experiments

When *Arctica islandica* was held (unfed) in circulating filtered seawater for 2 weeks, the color of the digestive gland changed from dark brown-green to pale brown. Within this time, tubules from all areas of the gland assumed a characteristic, uniform appearance comparable to Owen's (1972) "holding phase", which was characterized by three phenomena. First, the lumen of the tubule enlarged as the digestive cells changed from flask-shaped to low columnar or cuboidal. Secondly, the distal basophilic region of the digestive cells broadened and the β -metachromatic inclusions normally observed in type A digestive cells diminished markedly. Finally, the mid-cell inclusions lost some of their stainability; with Azan these inclusions stained less intensely with aniline blue than in fed quahogs, and the cyanophilia was limited to the periphery of the inclusion. However, the number of these inclusions in a digestive cell did not change markedly with starvation.

Approximately one-third of the *A. islandica* examined within one day of collection showed this typical holding phase morphology. After 7 days of starvation, histological examination of 10 animals showed noticeable flattening of the digestive cells; basal vacuoles were virtually empty after 14 days. However, two quahogs starved for over 2 weeks continued to show signs of fragmentation. With starvation, fragmentation can apparently occur more than once, ultimately resulting in the disintegration of the digestive tubules.

Aquadag was never phagocytized by the epithelium of the primary or secondary ducts, amoebocytes, basophils, or type B digestive cells; it was taken up by type A digestive cells in four of the nine specimens of *Arctica islandica* examined, in one case being phagocytized by these cells in large amounts within 25 min after feeding (Fig. 4). Selection by particle size occurred between the stomach and the digestive tubules. Graphite particles removed from the stomachs of four *A. islandica* were distributed in the following manner: $> 5 \mu\text{m}$, $< 1\%$; 2 to $5 \mu\text{m}$, 1% ; 1 to $2 \mu\text{m}$, 3% ; 0.4 to $1.0 \mu\text{m}$, 12% ; $< 0.4 \mu\text{m}$, 83% . Particles phagocytized by type A digestive cells were always from the smallest size class; these cells appeared unable to phagocytize particles larger than $0.4 \mu\text{m}$. In specimens of *A. islandica* fed suspensions of unicellular algae, intact algal cells or diatom frustules were never observed in sections of digestive cells.

Histochemical incubations were also performed on the digestive gland and stomach of quahogs which were starved for 7, 14, and 28 days. With two exceptions, incubation times similar to those used for unstarved animals yielded a strength and distribution of enzyme activity comparable to that observed in fresh animals. This observation was confirmed for the exopeptidase, endopeptidase, and esterase techniques, regardless of the length of starvation. However, in starved animals, acid phosphatase activity was not observed in digestive cells. Similarly, alkaline phosphatase activity was absent or barely detectable in basophil and digestive cells.

Starvation followed by feeding led to the appearance of acid phosphatase in the region of the digestive cell containing heterolysosomes (Owen, 1972). Also, alkaline phosphatase activity in the digestive tubules showed a correlation with the length of time which specimens of *Arctica islandica* were fed with mixed algal cultures at 5000 cells/ml. In quahogs starved for 4 weeks and then fed for 2 hr, the distal margin of the basophil cells showed a positive reaction for alkaline phosphatase activity (Fig. 5). In one of three animals tested, digestive cells were also weakly positive after 2 hr feeding. In *A. islandica* fed for 4 hr following prolonged starvation, the distal border of all digestive cells showed a positive reaction for alkaline phosphatase. In all cases, basophil cells were negative after 4 hr feeding. Most animals examined after 6 to 8 hr feeding showed a strong positive reaction for alkaline phosphatase along the basement membrane of the digestive tubules (Fig. 6). The basophil and digestive cells from these experimental animals were negative. In all phases of the feeding experiments, both primary and secondary ducts continued to show the strongest alkaline phosphatase activity in the gland.

DISCUSSION

Whole algal cells and graphite particles larger than $0.4\ \mu\text{m}$ were never phagocytized by the digestive cells of *Arctica islandica*; the histochemical results confirmed the importance of preliminary extracellular digestion in the stomach of this species. In this study, enzymes have been designated as extracellular when they were histochemically detectable both at the distal border of epithelial cells and in the lumen of the digestive gland or stomach. The principal source of these enzymes in *A. islandica* was the epithelium of the primary and secondary ducts. Although the distribution of several hydrolytic enzymes in the duct ceca closely paralleled that of the ducts, the strength of the histochemical reaction in the stomach wall was always much weaker. Further, large areas of the stomach, such as the posterior sorting area, were unreactive or only slightly reactive.

The function of the strong alkaline phosphatase activity in the duct epithelium is problematical. Alkaline phosphatase has often been implicated in phosphorylative transfer of extracellular molecules against concentration gradients at cell membranes (Rothstein, Meier, and Scharff, 1953; Hugon and Borgers, 1968; Monin and Rangueker, 1974). It could be argued that alkaline phosphatase activity in the duct cells indicates absorption, since autoradiographic studies have shown that the duct epithelium is an area of active absorption (Mathers, 1972). However, the level of alkaline phosphatase activity in duct cells was far stronger than

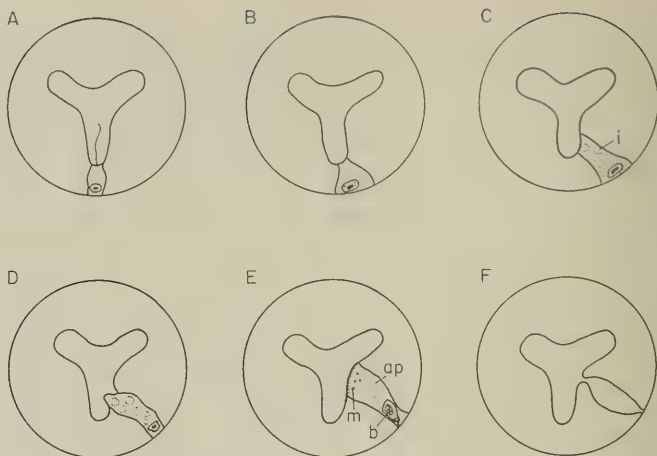


FIGURE 7. Six stages in the maturation of a digestive tubule cell: A, type A basophil cell; B, type B basophil cell; C, intermediate cell type; D, type B digestive cell; E, type A digestive cell; F, shedding of mature digestive cells. Region of acid phosphatase activity marked ap; b, basal vacuole; i, mid-cell inclusion; m, β -metachromatic inclusion.

that observed in digestive cells, which are known to be absorptive. Further, alkaline phosphatase was observed in the lumen of the ducts and duct ceca, an unexpected site for an enzyme supposedly involved in transfer of extracellular substances. It is possible that these enzymes are either utilized as extracellular hydrolytic enzymes or are involved in secretion of other hydrolytic enzymes at the cell membrane.

Although it has often been suggested that fragmenting digestive cells contribute to extracellular digestion in the lamellibranch stomach (Mansour, 1946; Morton, 1956), experimental proof has been lacking. In the present study, histochemical evidence from *Arctica islandica* supported Mansour's hypothesis. In the lumina of the tubules, ducts, and duct ceca, recognizable cell fragments were found which stained diffusely for esterases, endopeptidases, and acid phosphatases. Histochemical reactions for all of these enzymes were far stronger in the digestive cell fragments than in the epithelium of the ducts, stomach, or midgut. By means of mechanical action in the stomach, the influence of amoebocytes, or pH changes, these enzymes were released from the cell fragments to aid in extracellular digestion in the stomach. Neither cell fragments nor histochemical activity were recognizable in the midgut or in the feces.

The histochemical demonstration of endopeptidases in the stomach lumen is interesting in light of the discovery of significant cathepsin B activity in the stomach of the lamellibranch *Tresus capax* (Reid and Rauchert, 1976) and the septibranch

Cardiomya planctica (Reid, 1977). Cathepsin B, which in vertebrates is primarily concerned with intracellular protein turnover and autolysis, is apparently the endopeptidase most important in mediating bivalve intracellular proteolysis. It is reasonable to assume that the endopeptidase-like activity in the stomach of *Arctica islandica* is derived, by fragmentation, from the endopeptidase-like activity in the digestive cells.

If *Arctica islandica* and other bivalves utilize significant amounts of enzymes derived from burst fragmentation spheres, a reevaluation of the functional morphology of the ducts and duct ceca may be in order. While Morton (1956) noted that excretory spheres do break up in the stomach of *Lasaea rubra*, Owen (1966, 1973), because of morphological considerations, doubted that this was a common phenomenon in bivalves. Owen argued that the ciliated rejection tracts in the primary ducts and the disposition of the major typhlosole within the stomach served to isolate excretory spheres from the stomach contents. However, the efficiency of this isolating mechanism appears to vary significantly within the Bivalvia. The ciliated rejection tract is most strongly developed in anisomyarian bivalves (Owen, 1955), and it is in precisely these forms in which the typhlosole does not serve an isolating function (Owen, 1966). Conversely, in the order Gastropemta (Purchon, 1960), in which tongues of the typhlosole do penetrate the primary ducts, morphologically distinct rejection tracts in the primary ducts are either weakly developed (Owen, 1955), or, as in the case of *A. islandica*, absent.

The basophilic cells in the crypts of the digestive tubules have also been suggested as a source of extracellular enzymes. Summer (1969) identified β -glucuronidase and n-acetyl- β -glucosaminidase in the apical granules of these cells in *Mytilus edulis*, and Mathers (1973) found that esterases and exopeptidases were secreted by the crypt cells of *Crassostrea angulata* and *Ostrea edulis*. In *Arctica islandica*, however, the only enzyme identified in the region of the apical granules was alkaline phosphatase, and even that was observed only sporadically and in moderate concentrations. Of course, it is possible that other hydrolytic enzymes are stored in the apical granules in an inactive form, awaiting activation in the lumen of the tubule. This is the case with trypsin found in the pyloric ceca of starfish (Hori, Tanahashi, and Matsuoka, 1977).

After over 50 years of study, the function of the cell types in the bivalve digestive gland remains controversial. All of the ultrastructural evidence indicates that basophilic cells are specialized for enzyme secretion (Summer, 1966b; McQuiston, 1969; Owen, 1970; Pal, 1971). This interpretation, however, is clouded by several pieces of contradictory evidence. First, a micrograph showing fusion of secretory granules with the cell membrane has apparently never been obtained (Pal, 1971). Until such a micrograph is published, there is no assurance that "secretory" granules are actually secreted. Further, histochemical studies have failed to demonstrate either the nature or the importance of apical granules of basophilic cells. Finally, the breakdown of type A digestive cells make a replacement mechanism for them necessary. In the digestive tubules, mitosis only occurs in type A basophilic cells, which suggests their ultimate importance in replacement of digestive cells.

A number of models have been proposed synthesizing this somewhat contradictory information. Pal (1971) concluded that most of the protein in the

apical granules of *Mya arenaria* basophils is not secreted, but autodigested. Owen (1970) described three cell types in the digestive tubules of *Cardium edule*—mature digestive cells, mature (basophilic) secretory cells, and immature flagellated cells; the latter may replace either or both of the other two. Fankboner (unpublished, in Owen, 1972) found stem cells in *Tridacna* which give rise to both mature flagellated cells and pyramidal cells. The latter are transitional between stem cells and digestive cells. He also suggested that the apical organelles of the pyramidal cells are not secretory granules, but rather primary lysosomes.

Cytological and histochemical evidence suggests that, in the digestive tubules of *Arctica islandica*, there is only one basic cell type, which passes through a number of distinct phases in its life history (Fig. 7). Type A basophils divide frequently and serve as primary generative cells. These are the only cells in the tubules which show evidence of mitosis. They mature into type B basophils by the development of extensive granular endoplasmic reticulum and characteristic Golgi apparatus, organelles necessary for the elaboration of primary lysosomes and heterolysosomes. The latter, after all, appear to be preformed vesicles found in all digestive cells, regardless of feeding state (Owen, 1972; Thompson, Ratcliffe, and Bayne, 1974). Type B basophils pass rapidly through intermediate cell types, characterized by both basophilia and digestive vesicles, to form immature or type B digestive cells.

Previous workers have treated the digestive cell as a single functional cell type (Yonge, 1926b; Pal, 1972). In *Arctica islandica*, however, type A and type B digestive cells are clearly distinguishable, much as type A and type B basophils are, as morphologically and functionally different stages of a single cell type. Type B digestive cells have an oval, well-formed nucleus. These cells, however, lack basal vacuoles (which are probably compound residual bodies), β -metachromatic phagosomes, and the ability to phagocytize particulate matter in the size range 0.1 to 0.5 μ m. Type B digestive cells develop into type A or mature digestive cells, located centrally on ridges of digestive tubules. Mature type A digestive cells have a prominent basal vacuole, and compressed, stellate nuclei. They also contain distal β -metachromatic phagosomes, are able to phagocytize 0.1 to 0.5 μ m particulate matter, and show the strongest intracellular reaction for acid phosphatase of any cell type in the digestive gland. It is clearly the type A digestive cells which perform the bulk of intracellular digestion in *A. islandica*. Excretion by these mature digestive cells takes two forms. Small pieces of cells may be nipped off without cell death, or entire, mature cells may be shed. It is likely that a single digestive cell releases wastes several times before it ages. Ultimately, the basal vacuole and the cell fill with debris, the nucleus disintegrates, and the individual cell falls away from the basement membrane, in time to be replaced by the dividing basophilic cells in the crypts.

If immature basophilic cells serve to replace both secretory and digestive cells, as Owen (1970) suggested, it is difficult to imagine how immature cells could replace shed digestive cells in a well-formed digestive tubule, unless stem cells were located between digestive cells on the tubule ridges. This arrangement, however, was never observed. Further information on the function of the cell types in the bivalve digestive gland will come with thymidine-labelling studies (Owen, 1972) and enzymatic analysis of specific organelles (Hori *et al.*, 1977).

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SUMMARY

1. The histology and enzyme histochemistry of the stomach and digestive gland of *Arctica islandica* were examined. Several enzymological and morphological features contribute to a well-developed system of extracellular digestion in this species.

2. Extracellular exopeptidases and alkaline phosphatases are largely derived from primary and secondary ducts. The epithelium of these ducts also secretes esterases and acid phosphatases. In all cases, secretory activity of the ducts overshadows that of the stomach wall. Other digestive enzymes, including acid endopeptidases, are supplied by fragmenting digestive cells. Starvation has little apparent effect on most enzyme activity, but does depress secretion of alkaline phosphatases and intracellular production of acid phosphatases. These enzymes can be induced under appropriate feeding conditions.

3. The primary ducts in *A. islandica* are organized into many ciliated folds, rather than separate rejection and acceptance tracts characteristic of anisomyarian bivalves. This structural arrangement insures that enzymes present in digestive cell fragments are utilized in the gastric cavity, rather than being rejected by the intestinal groove.

4. Mature and immature basophilic cells, intermediate cells, and mature and immature digestive cells are present in the digestive tubules. The two types of digestive cells are distinguishable on the basis of cellular inclusions, ability to phagocytize particulate matter, metachromatic staining, and nuclear structure.

5. Cytological and histochemical evidence suggests that, in the digestive tubules of *A. islandica*, there is only one basic cell type, which passes through a number of distinct phases in its life history.

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ASPECTS OF DEVELOPMENT AND OCCURRENCE OF
CARCINONEMERTES EPIALTI (NEMERTEA)
FROM SHORE CRABS IN MONTEREY BAY,
CALIFORNIA

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Nemerteans of the genus *Carcinonemertes* are symbiotes of various species of crabs (Coe, 1902; Humes, 1942; Kuris, 1978; Wickham, 1978). Although juvenile worms are found on males and immature females, the worms grow to maturity and reproduce only on female crabs brooding eggs (Humes, 1942; Kuris, 1978; Roe, personal observation). When the worms are abundant they can be important egg predators, consuming large numbers of host eggs (Wickham and Fisher, 1977). *Carcinonemertes errans* living on *Cancer magister* is an important causative factor in fluctuations of abundances of this commercially important edible crab along Central and Northern California (Wickham, Bodega Bay Marine Laboratory, personal communication).

The reproductive success of the nemerteans is closely linked to the reproductive biology of the crab and can also be important to the reproductive success of the crab. This study concerns some aspects of the reproductive biology of *Carcinonemertes epialti* on the grapsoid crabs, *Hemigrapsus oregonensis* and *Pachygrapsus crassipes* and aspects of the distribution of *C. epialti* on these crabs in central Monterey Bay, California.

MATERIALS AND METHODS

Each month for nearly two years, approximately 25 individuals of *H. oregonensis* and 25 individuals of *P. crassipes* were collected during low tide from under boulders near the southwest edge of the U. S. Highway 1 bridge at Elkhorn Slough. Twenty-five individuals of *P. crassipes* were also collected nearly every month at low tide from under boulders at Carmel Point, Monterey Bay, for 1 year. Crabs were brought to the laboratory and were maintained at approximately 15° C. Each crab was searched for worms within 24 hr of collection. The number, position and general size of the worms on each crab was determined in the laboratory with the aid of a dissecting scope. Length and width of the carapace of each crab was measured and these figures were multiplied to get an estimate of crab area. Most crabs were also frozen and the carapace was then removed for observation of the number and position of worms found under the carapace. These are called "internal" worms although they are not actually within the internal organs of the crab (see Kuris, 1978). Since crabs were not dissected every month, and since some months a few crabs were kept alive for observations, only worms seen without dissecting the crab were used for infestation rates, etc., unless otherwise stated.

Data were divided into "summer" (April–September) and "winter" (October–March) seasons to correspond to the two breeding seasons of *H. oregonensis* (Kuris, 1978; Roe, personal observation). These divisions also roughly correspond to the one summer breeding season of *P. crassipes* (Hiatt, 1948; Roe, personal observation).

Adult female worms are much longer (4–6 mm) than juveniles (to 1 mm). Males and immature females are intermediate in size. In this paper females and juveniles were recognized by size; medium-sized worms were not assigned a sex.

For observations on reproductive biology, worms were removed from crabs and placed singly or in pairs into standard 10.4 cm diameter fingerbowls containing clean, unfiltered sea water and were maintained at 15° C. They were observed at intervals with the use of a dissecting scope. Worms will lay egg masses on bottoms and sides of fingerbowls, and since it is much easier to follow development of eggs not entangled among crab eggs, most observations of development were done with worms thus separated from their hosts. From observations of behavior of worms found naturally on crabs and from observations of worms experimentally transferred to crabs, worms removed from crabs and placed into fingerbowls appear to behave as in nature.

Using animals maintained in fingerbowls, the number of egg masses made by each female, when and where on the fingerbowl each egg mass was placed, size of the eggs, size of the egg masses, number of eggs per egg mass, time between laying and hatching, time between the start of hatching and emptying of an egg mass, length of time larvae lived after hatching, and the length of time adults lived after laying eggs were observed. To study starvation, one worm was placed into clean, unfiltered sea water in a fingerbowl and was observed periodically until dead or until it could not be found during two consecutive observation periods. To study growth and aspects of reproductive biology of worms on crabs, worms were transferred to cleaned brooding female, male, and nonbrooding female crabs and were observed. Natural worm populations on isolated male, female, and brooding female crabs were also observed through time.

RESULTS

Worms found naturally on brooding female crabs laid their egg masses among the crab eggs (two laid eggs 2 days after first observation); many adult worms and egg masses were observed on brooding crabs from nature (see also Coe, 1902; Humes, 1942; Kuris, 1978). Some small worms that were transplanted to the egg masses of brooding crabs similarly grew, matured, and laid egg masses. In six experiments of transferring worms to brooding *H. oregonensis*, two observations of the natural worm population on brooding *H. oregonensis*, and one observation of the natural worm population on brooding *P. crassipes*, most (40 from a total of 56 worms) were lost (died or became unobservable) or showed no growth and did not lay eggs. However, 9 of 15 juveniles placed on one crab grew to adult female size in 14 days and three others did so in 27 more days. In three other experimental transfers, four worms grew and became mature within 17 days and each laid an egg mass within 25 days of transfer to the crab.

In contrast to the worms that grew and laid eggs on some of the brooding crabs, no worms experimentally placed on male or nonbrooding crabs of either species ever grew or laid eggs (50 worms observed in 5 experiments). Those worms found naturally on male or nonbrooding female crabs and observed through time also did not grow or reproduce (over 34 worms in 4 observations). These transfer experiments show that worms mature and lay eggs only on ovigerous female crabs and that the experimental worms behave similarly to worms observed from nature.

In fingerbowls, 11 adult female worms from *H. oregonensis* laid an average of 2.8 egg masses/female (range = 1–5 egg masses/female, s.d. = 1.47), 1 about every 2.4 days (range = 1 egg mass/day to 1 in 8 days). No female laid eggs for more than 12 days. Four female worms from *P. crassipes* laid an average of 3.5 egg masses (range = 1–6/female, s.d. = 2.08) per worm, each an average of every 2.1 days (range = 3 masses in 2 days to 1 mass in 5 days) for up to 13 days of egg laying.

Egg masses were usually elongate tubes about as wide as the worm, were made of a mucus-like substance, and adhered to the dish by mucus-like strands from various places along the length of the mass. Egg masses of worms from *H. oregonensis* had an average of 81 eggs per egg mass (14 egg masses counted, range = 8–189 eggs, s.d. = 59.42). These egg masses were an average of 3.03 mm in length (range = 2 mm–4.2 mm, s.d. = 0.94). Eggs in the egg masses were oval, 7 to 7.5 μ by 6 to 6.5 μ diameter.

Within 24 to 37 hr after being laid, larvae were gastrulae. They gradually developed two eye spots and cilia, and began rotating within the mucous egg case 4 to 7 days after the egg mass was laid. Finally, each would break loose or swim out either end of the egg mass or any other opening made by other larvae. Larvae swim smoothly by cilia. Fully developed larvae have an anterior and posterior flagellar region apparently made of two to four flagella each. The anterior flagella rotate while the larva is swimming; the posterior, shorter flagella are usually held straight out posteriorly and appear not to bend during swimming. In this study it took an average of 6.6 days (range = 2–10, s.d. = 2.06, 18 samples) for larvae from *H. oregonensis* and 9 days (range = 6–11 days, s.d. = 1.34, 11 samples) for larvae from *P. crassipes* to start hatching from the egg mass. There was a tendency for eggs in cases laid later in the laying period to hatch within fewer days than those laid earlier.

Not all larvae hatch at once from an egg mass; rather, they break free a few at a time, often first at one of the ends of the case. The average time from the start of hatching until an egg case was empty was 12.8 days (range = 4–22 days, s.d. = 6.40, 8 samples) for worms from *P. crassipes*. Only one egg mass was thus followed from worms from *H. oregonensis*; it became empty in three days.

Larvae gradually elongate and begin to develop internal organs. They swim more and more slowly and closer and closer to the bottom. It is assumed from studies done on development of *Paranemertes peregrina* (Roe, 1976) whose larval development pattern is similar, that metamorphosis is gradual. Larvae from *H. oregonensis* lived in fingerbowls without any food except contaminate bacteria and ciliates an average of 21.25 days ($N = 8$ samples, range = 10–50

days, s.d. = 13.60). Only one sample of larvae from *P. crassipes* was followed until all larvae died. They lived 28 days. The longest any larva lived under these conditions was 50 days.

Whether adult worms die after egg laying or whether they stop feeding, regress in size and wait until the crab broods more eggs to repeat their own egg-laying cycle is unknown (see also Kuris, 1978). In the experiments with worms that laid eggs in fingerbowls, worms from *H. oregonensis* became wrapped in mucus and tun-shaped in an average of 17.2 days ($N = 6$, range = 11–20 days, s.d. = 3.43); most were dead in 22 days. Four worms from *P. crassipes* became tuns in an average of 16.5 days (range = 9–20 days, s.d. = 5.20); a fifth died in 21 days. In comparison, in starvation experiments with five juvenile (1 mm) and five medium-sized (2–4 mm) immature worms from *H. oregonensis*, the juvenile worms were quiescent in an average of 24.7 days (range = 13–27 days, $N = 3$, s.d. = 10.69) and were dead in an average of 35.2 days ($N = 5$, range = 20–58 days, s.d. = 17.12). The medium-sized worms were quiescent in an average of 50.8 days ($N = 4$, range 23–68 days, s.d. = 21.69) and were dead in an average of 72.8 days ($N = 5$, range = 30–113 days, s.d. = 29.58).

Although larvae lived many days and developed to some extent, actual metamorphosis could not be induced (with one possible exception; see below). In all experiments with crab zoea introduced to fingerbowls containing worm larvae, no small worms were ever found on the zoea (three experiments with many zoea, many worm larvae). In exposures of 21 crabs of both species and sexes to many larvae, only one showed any evidence of metamorphosed larvae: two tiny worms were found on a brooding female *H. oregonensis* 22 days after she had been cleaned of worms, but whose worm egg masses and swimming larvae were left among her eggs. However, it is possible that these two tiny worms were missed in the cleaning, so these results are inconclusive.

From studies of natural occurrence of worms on crabs, it was found that at Elkhorn Slough over a period of 19 months between June 1975 and April 1977, 72.21% of the *H. oregonensis* population harbored *C. epialti* with an average of 14.56 worms per crab. Brooding crabs had the highest infection rate with 90.44% carrying at least one worm, and an average worm load of 19.49 per female. (Figures include crabs without worms.) Sixty-seven per cent of the male crabs had worms, with an average load of 17.13 worms/crab; 61.87% of the nonbrooding female crabs had worms, with an average of 6.05 worms/crab.

Infection rates of *P. crassipes* at Elkhorn Slough were much lower than were those for *H. oregonensis*. Overall infection rate from July 1975 to April 1977 was 47.47% with an average worm load of 3.34 worms/crab. Again, the part of the population which had the highest proportion of worms was brooding females, with an average of 51.06% infected and an average of 1.3 worms/crab. Forty-nine per cent of the males had worms, with an average of 4.45 worms/crab. This worm load reflects the high number of worms present at Elkhorn Slough during winter months. During summer, the only season in which brooding *P. crassipes* occurred, the average worm load for males was only 0.78 worm crab. Of nonbrooding females, 44.64% had worms, with overall average of 2.55 worms/crab and a summer average of 0.22 worm/crab.

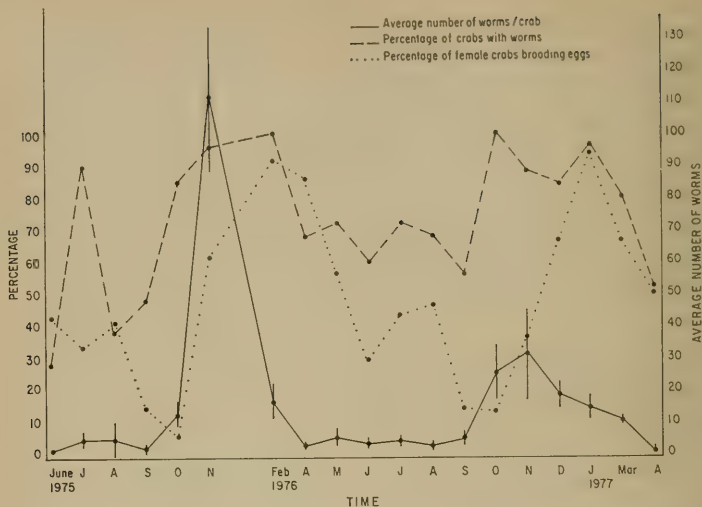


FIGURE 1. Percentage of *H. oregonensis* population at Elkhorn Slough, California harboring *C. epialti* externally, average number of worms/crab, and percentage of females of the crab population that were brooding eggs each month. Vertical lines along the solid line are one standard error from the mean. For most months, sample size was 25 crabs (exceptions: July 1975, $N=20$; August 1975, $N=29$; October 1975, $N=26$). The overall infection rate was 72.21%. Overall average number of worms/crab was 14.56 ± 4.14 .

Although *P. crassipes* was collected at Carmel Point for 10 months (July 1975–July 1976), no specimens of *C. epialti* were found on any crab from there, nor were any adult brooding female crabs found at that site.

One-way analysis of variance tests showed that for both species of crabs at Elkhorn Slough the infection rate for females with eggs was significantly greater than for males or nonbrooding females during both breeding seasons of *H. oregonensis* (F , 269 crabs, April–September = 31.4998, $P < 0.001$; F , 195 crabs October–March = 8.0694, $P < 0.001$) and during the one breeding season for *P. crassipes* (F , 214 crabs, April–September = 8.2744, $P < 0.001$). During winter when there were many worms present, there was no significant difference between infection rates of male or nonbrooding female *P. crassipes* (F , 184 crabs, October–March = 1.1812, $P > 0.90$), but there was a significant difference between male and nonbreeding female infection rates in summer with males having a significantly higher infection rate than females (F , 175 crabs = 8.0877, $P < 0.001$).

Since the number of worms found on a crab was divided by a measurement of crab carapace area to get the number of worms/unit area of crab for analysis of variance, size of the crabs was not a variable. Large crabs often have more

worms than small ones, but they also have more area to harbor worms and from results of the analysis of variance tests, it appears that worms are not selecting crabs on the basis of size.

From data on the per cent of infected crabs, the average number of worms/crab, and the analysis of variance results, it can be seen that in both species females with eggs are most heavily infected, followed by males, then by females without eggs. There was a strong seasonal component to both the infection rate and the number of worms per crab in both populations of crabs (Figs. 1, 2) and in all groups of crabs. The worm population was highest from October to January with sharp peaks in November. The peaks in worm population precede the winter reproductive period of *H. oregonensis* (Fig. 1) which is the major reproductive period for this crab species (Kuris, 1978; Roe, personal observation). The fall peak in the worm population does not correspond at all to the (summer) reproductive cycle of *P. crassipes* (Fig. 2); indeed, most of the infection of this species is also in fall and winter (Fig. 2) even though females brooding eggs are the most heavily infected hosts when they are present.

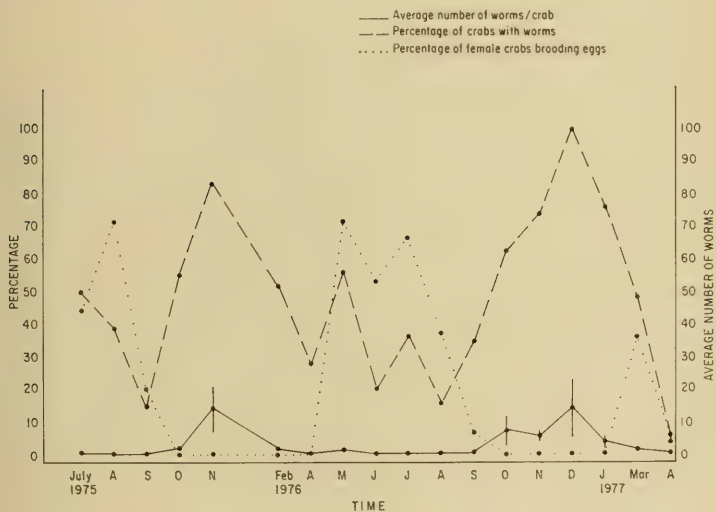


FIGURE 2. Percentage of *Pachygrapsus crassipes* population at Elkhorn Slough, California, harboring *Carcinonemertes epialti* externally, average number of worms/crab, and percentage of females of the crab population that were brooding eggs each month. Vertical lines along the solid line are one standard error from the mean and are shown only when the standard error exceeded 1.0. For most months, sample size was 25 crabs (July 1975, N = 14; August 1975, N = 18; September and October 1975, N = 20; November 1975, N = 24; July 1976, N = 22; October 1976, N = 24; November 1976, N = 23). The overall infection rate was 47.47%. Overall average number of worms/crab was 3.34 ± 0.88 .

The overall infection rate of *P. crassipes* when there were brooding females was 33.55%; in months when there were no brooding females, 66.34% of the crabs were infected. The number of crabs infected versus the number not infected between March to September and October to February was significantly different ($\chi^2 = 58.25$, $P < 0.005$) even when brooding females were included in summer months.

The worm population found on male and nonbrooding female crabs consisted almost entirely of juveniles. From a worm population of 6918 individuals on *H. oregonensis*, only 2 large and 1 medium-sized worms were ever found on males; 10 large and 9 medium-sized worms were found on females without eggs. All other individuals found on males or nonbrooding females were juveniles. In contrast, 772 worms, or 28.89% of the worms found externally on brooding crabs, plus another 6 found internally, were large females. When the number of crabs with and without adult female worms were compared, brooding crabs had significantly more adult worms than all other crabs combined ($\chi^2 = 233.55$, $P < 0.005$). Of the worms found on brooding *P. crassipes*, 26.67% were large enough to be considered mature or nearly-mature female worms. Again, the number of brooding crabs with adult female worms was significantly greater than all other crabs combined ($\chi^2 = 38.697$, $P < 0.005$); no large worms were found on specimens of *P. crassipes* that were not brooding eggs.

Only 16 adult female worms were found on *P. crassipes*, comprising only 1.15% of the worm population found on those crabs. In contrast, 790 individuals, or 11.42%, of the total worm population found in or on *H. oregonensis* was mature or nearly-mature females. August and September and early spring were periods of a greater per cent of female worms in the worm population of *H. oregonensis* (Fig. 3) and in late summer the proportion of adult female to other worms on brooding crabs was highest (Fig. 3).

Worms were primarily found in the axillae between crab legs and under the abdominal flap, either on the abdomen itself or on the central surface of the thorax (see also Kuris, 1978). A few worms were also found on the chelipeds. When the crab carapace was removed, worms were found mainly on the pericardial sacs posterior to the gills. Only one juvenile worm was ever found on the actual gills. There was a different distribution of worms on brooding crabs, nonbrooding females, and males in both species of crabs. Nearly all worms (95.2% in summer, 96.1% in winter in *H. oregonensis* and 88.5% in *P. crassipes*) were found under the abdominal flap, either on the thorax or at various sites of the abdomen, but in close proximity to the crab eggs on brooding females. Nonbrooding females included a range of crabs from immature females to ones that had recently released zoea. Those that had recently released zoea probably had a concentration of worms under the abdomen. Nonbrooding females had significantly more worms under the abdomen than on the legs (*H. oregonensis*, 39.2% in winter, $F = 4.5016$, $P < 0.001$; 48.6% in summer, $F = 4.1243$, $P < 0.001$; *P. crassipes*, 42% in winter, $F = 3.6542$, $0.005 > P > 0.001$; *P. crassipes*, 93.3% in summer, $F = 9.5277$, $P < 0.001$), but the clumping was not as great as with brooding females. Males had a fairly even distribution of worms between the legs and under the abdomen and there was no significant accumulation of

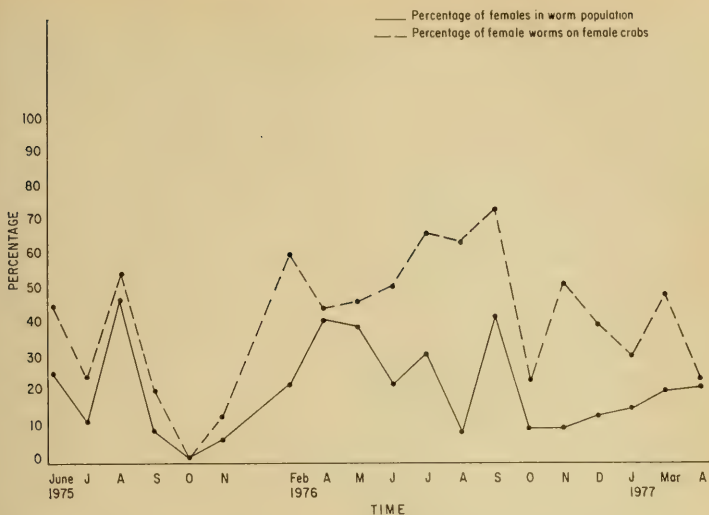


FIGURE 3. Percentage of the *C. epialti* population comprised of mature or nearly-mature females and percentage of the worms found on brooding females of *H. oregonensis* that were mature or nearly-mature females. Overall percentage of worm population that was female was 11.42%. Overall percentage of the worms found in female crabs that were females was 28.89%.

worms at any site, except that worms on *H. oregonensis* were clumped under the abdomen in summer (*H. oregonensis* in winter, $F = 1.9957$, N.S.; in summer, $F = 5.3227$, $P < 0.001$; *P. crassipes* in winter, $F = 1.9300$, N.S.; in summer, $F = 1.8017$, N.S.). The distribution of worms on male, nonbrooding, and brooding female crabs was significantly different both summer ($\chi^2 = 329.8407$, $P < 0.005$) and winter ($\chi^2 = 1719.3225$, $P < 0.005$) for *H. oregonensis* and in summer ($\chi^2 = 71.4088$, $P < 0.005$) and winter ($\chi^2 = 34.8498$, $P < 0.005$) for *P. crassipes*, when the number of worms on all legs versus the combined number on the thorax and abdomen were compared for the three types of crabs (except there were no brooding females in the winter *P. crassipes* sample).

DISCUSSION

Results of this study show that the highest population of worms occurred in November just preceding the major winter reproductive period of *H. oregonensis* (Fig. 1). The large numbers of worms found in fall were primarily juveniles and the highest percentages of female worms occurred in August–September and April (Fig. 3). It appears that there is a major reproductive period for

C. cpialti during late summer (Fig. 3) when there are brooding individuals of both *H. oregonensis* and *P. crassipes* present. There also appears to be another smaller reproductive peak for the worms in April (Fig. 3) and there is probably some reproduction nearly every month of the year at Elkhorn Slough, since brooding females of *H. oregonensis* could be found every month (Figs. 1, 2).

Periods of high percentages of female worms in the population (especially August–September; Fig. 3) are followed by increased numbers of worms, mainly juveniles, per crab (Figs. 1–3) about 2 months later. The stimulus for metamorphosis of *C. cpialti* is unknown, but larvae are not found on crabs (see also Kuris, 1978). However, from the present data, it appears that larvae probably settle directly onto crabs or at least do not spend much time elsewhere in their environment since juvenile increases in worm populations followed within 2 months of reproduction, and since this is well within the 24 to 85 day period larvae can live without metamorphosis. I suggest that these worms have approximately a six-month life span and that the Elkhorn Slough populations have two major reproductive periods each year. In starvation experiments, no worm lived more than 113 days with averages of 22 days for adults after egg laying, 72 days for medium-sized worms, and 35 days for juveniles after the experiments were started. Of course, presumably worms can live much longer under optimal conditions. If this species is found to have a short life span, it will differ considerably from *C. carcinophila*. *Callinectes sapidus*, the major host of *C. carcinophila*, does not molt after reaching maturity and worms apparently migrate to the gills to feed while not reproducing (Humes, 1942). Coe (1902) thought it probably took up to a year for *C. carcinophila* to reach maturity and, under the circumstances, it would be adaptive to have a longer life span for that species.

C. cpialti apparently has a wide range of hosts (Kuris, 1978; Coe, 1902; Wickham, 1978). From this study it appears that the same species of worm lives on both species of shore crabs studied but the life history of *C. cpialti* is much more closely tied to the life history of *H. oregonensis* than to that of *P. crassipes*. A much larger percentage of the former were infected (72% vs. 47.5%) and they had a higher average number of worms/crab (14.6 vs. 3.3). Also, only 1.15% of the worm population found on *P. crassipes* were adult females compared to 11.4% of the *H. oregonensis* population. Finally, a significantly greater percentage of the *P. crassipes* population was infected in winter than in summer even though *P. crassipes* has only one breeding cycle per year (during summer; Hiatt, 1948; Fig. 2) and when they were present in summer, the brooding females were the most heavily infected members of the *P. crassipes* population. Kuris (1978) never found *P. crassipes* with *C. cpialti* at Bodega Bay, nor were any found on this crab at Carmel Point in the present study.

Furthermore, with respect to timing of reproductive and developmental events, *C. cpialti* appears better adapted to *H. oregonensis* than to *P. crassipes*. In *H. oregonensis* females brood eggs 41–43 days and zoea hatch on the 44th day (Kuris, 1971, 1978). Worms living on *H. oregonensis* lay eggs during the last 23 to 26 days of this period (Kuris, 1971). A female typically takes 17 days (24–30 days in Kuris' study) to mature. Then she lays about three egg masses in the next 12 days. Full development of the embryo takes an average of 6.6 days and it takes another 3 (one observation only), or an average of 13 days

(using data from worms from *P. crassipes*), to empty the egg cases. Larvae can survive an average of 21 days. On the average then, development of females and larvae takes about 27 to 37 days plus the time larvae live in the plankton. There is wide latitude in this range, however; the time from egg laying to larval death without metamorphosis (an average of 31–41 days) can range from 24 days to a maximum of 84 days.

P. crassipes has a brooding period of 26 to 31 (average = 29) days (Hiatt, 1948). Worms from *P. crassipes* in this study typically laid 3.5 egg masses in 13 days. It took an average of 9 days from egg deposition to hatching and an average of 13 days to empty the egg cases. With the 17 days (at least) that it takes to mature, development of females and larvae takes an average of 39 days for the reproductive cycle to be complete. Larvae lived 28 days. Length of larval life then could range from a minimum of 35 days to a maximum of 85 days. Although *H. oregonensis* females brood their eggs longer than do those of *P. crassipes*, the reproductive cycle of the worms on *P. crassipes* is slightly longer than in *H. oregonensis*.

It is not known whether *C. epialti* prefers *H. oregonensis* to *P. crassipes* or whether some aspect of the life history of *P. crassipes* prevents very successful habitation by *C. epialti*, but both life history events and density of worms suggest they are better adapted to *H. oregonensis* than to *P. crassipes*.

I wish to thank Diane Davies for much help with field and lab work on this project. I also wish to thank Carolyn Argo for typing the manuscript and Jay Christofferson, Dan Wickham, Armand Kuris, and Cadet Hand, who provided many helpful suggestions and Louis Feldman who helped with statistics. Thanks to the personnel at the Moss Landing Marine Laboratories for use of facilities after field trips.

SUMMARY

1. In the laboratory, females of *Carcinonemertes epialti* from *Hemigrapsus oregonensis* laid an average of 2.8 egg masses, one about every 2.4 days, for 12 days. Larvae started hatching in 6.6 days and lived an average of 21 days and a maximum of 50 days without metamorphosis.

2. Females of *C. epialti* from *Pachygrapsus crassipes* laid an average of 3.5 egg masses, one every 2.1 days, for 13 days. Larvae started hatching in 9 days. It took an average of 12.8 days for all larvae to emerge from egg masses. Larvae lived an average of 28 days without metamorphosis.

3. Egg masses were an average of 3.03 mm long and contained an average of 81 eggs per mass.

4. Adult worms lived about 22 days without food after egg laying ceased. Medium-sized starving worms lived about 73 days and starving juveniles lived 35 days in comparison.

5. In nature, 72.21% of the *H. oregonensis* population was infected, with an average of 14.56 worms/crab; 47.47% of the *P. crassipes* population was infected, with an average of 3.34 worms/crab.

6. In both crab populations brooding females were most heavily infected both in per cent infected and in average worm burden. These were followed by males, then nonbrooding females.

7. About 29% of the worms found on brooding *H. oregonensis* and about 27% of those found on brooding *P. crassipes* females were adult females. Peaks in abundance of adult female worms occurred in August–September and early Spring.

8. Worms were distributed primarily under the abdomen close to eggs of brooding female crabs. More worms were found at this location than at other sites on nonbrooding females; on males, worms were fairly evenly distributed in the axils between legs and on the abdomen.

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Continued on Cover Three

THE BIOLOGICAL BULLETIN

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THE CYTOCHEMISTRY OF *LIMULUS* EGGS

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Much of the accumulated literature on the cytochemistry of female gametes has been concerned with the chemical composition of the compound yolk produced during oögenesis (Nath, 1968). Egg envelopes have received somewhat less attention in comparison. While echinoderm and amphibian egg jellies have been of special interest to many investigators (Monroy, 1965), it has been the egg investments of economically important organisms that have been studied extensively (Monne, 1959; Smyth and Clegg, 1959). Arthropod eggs have been the subject of numerous investigations but the bulk of these investigations has been concerned with development and physiology, not cytochemistry. Among arthropod eggs, the insect egg has been the favored material for such investigations.

Limulus polyphemus L., an arthropod commonly known as the horseshoe crab, has been extensively used in embryology (Brown and Knouse, 1973) and as a result the *Limulus* egg has been adequately described in the scientific literature. Two extensive accounts on oögenesis in *Limulus* by Munson (1898) and Gardiner (1927) appeared some years ago and more recently, Dumont and Anderson (1967) conducted an ultrastructural study on oöcyte maturation in *Limulus*. In addition, transmission and scanning electron micrographs showing the structure of the egg envelopes have been presented in studies on sperm-egg interactions (Shoger and Brown, 1970; Brown and Humphreys, 1971). Even though much is known about the morphology of the egg, very little information exists in the literature on the chemical nature of the *Limulus* egg. This study is therefore an investigation of the chemical nature of the egg envelopes, cortex and yolk of the *Limulus polyphemus* egg.

MATERIALS AND METHODS

Specimens of male and female *Limulus polyphemus* L. were obtained from the Florida Marine Biological Specimen Company, Panama City, Florida, and from

the Supply Department, Marine Biological Laboratory, Woods Hole, Massachusetts. The animals were kept in Model 250 Instant Ocean Culture Systems at a temperature of 15° C for periods up to six months before discarding. Approximately 90 animals were used during the course of this study.

Collection and preparation of gametes

Spermatozoa and eggs were collected by electrical stimulation with an Adjust-A-volt electrical stimulator. A setting of 30 volts on the stimulator was maintained at all times. A transformer was positioned between the electrodes and the stimulator in order to obtain a potential of 3.8 volts. The procedure of collecting spermatozoa and eggs involved placing the animal on its dorsal surface, lifting the genital operculum while depressing the gill opercula, and applying the electrodes to the gonopore region. Spermatozoa were collected with disposable Pasteur pipets and subsequently transferred to graduated centrifuge tubes for use in insemination studies. Routinely 10% sperm suspensions containing approximately 10^9 spermatozoa/ml were prepared with cold (5°–15° C) filtered Instant Ocean synthetic sea water or Marine Biological Laboratory (MBL) synthetic sea water (Cavanaugh, 1956) for use in insemination studies. Since freshly spawned eggs adhere to glass or metal, special care was taken during handling. Eggs were collected from the gonopore region of the female with a Parafilm "M" covered tablespoon. Eggs to be used in insemination experiments were placed in Parafilm "M" lined petri dishes or in plastic petri dishes containing cold sea water. Eggs to be used in cytochemical and biochemical experiments were washed from the tablespoon with cold filtered sea water into a plastic test tube. All subsequent manipulations of fresh eggs were carried out with wood applicator sticks.

Preparation of uninseminated eggs for cytochemistry

Following collection and three cold sea-water washes, *Limulus* eggs were immediately frozen on a specimen holder at -20° C in an International Cryostat CT-1, sectioned at 12 μ and collected on cold glass microscope slides or 22 mm sq. cover glasses, fixed at 0° C in 10% neutral buffered formalin (Thompson, 1966) for 1–12 hr and washed in running tap water for 1 hr. Microslides containing egg sections not immediately being used were stored in plastic slide boxes at -25° C. These stored egg sections were normally used within a week. Other eggs were collected and placed directly into 10% neutral buffered formalin (Thompson, 1966) at room temperature (23° C). To ensure fixation, the eggs were punctured with fine point forceps and a dissecting needle after 15 min in the fixative. Punctured eggs were placed in fresh fixative for a period of 48 hr and then washed in running tap water overnight. The eggs were subsequently dehydrated in a graded series of ethyl alcohols, cleared in benzene or xylene, embedded in Paraplast (56°–57° C), sectioned at 8–10 μ on an American Optical Model 820 microtome, and prepared for cytochemical methods. The distribution and types of carbohydrates, proteins, amino acids, lipids, nucleic acids and phosphatases were determined on uninseminated *Limulus* egg sections. A summary of the cytochemical methods and results is listed in Table I.

TABLE I

Cytochemical reactions of uniseminated Limulus eggs.*

Stain or test	Basement lamina	Reaction		Yolk
		Vitelline envelope	Cortical region	
Periodic-acid Schiff (PAS)	—	+++	++++	++++
Potassium Permanganate Schiff (PPS)	+	+++	++++	++++
Chromic Acid Schiff (CAS)	—	—	+	+
Congo red	++	+	+++	+++
Alcian blue (pH 1.0)	—	—	+++	—
Alcian blue (pH 2.5)	—	—	+++	—
Toluidine blue 0	—	—	++	—
Azure A	—	—	—	—
Periodic acid-paradiazine (PAD)	—	++ (gray-brown)	++ (brown)	++ (brown)
Mild methylation Alcian blue (pH 2.5)	—	—	+	—
Methylene blue extinction				
pH 2.6	—	—	—	—
pH 3.6	—	—	—	—
pH 4.3	—	+	+	+
pH 5.3	—	+	+	+
pH 6.7	++	++	++	++
pH 7.6	++	+++	+++	+++
pH 8.1	++	++++	++++	++++
Mercuric bromphenol blue	++++	++++	++++	++++
Performic acid-Alcian blue	—	—	++	+
Ninhydrin-Schiff	++++	+++	++	++
Sekiguchi method	—	+	+	+
Naphthol-yellow S	+++	+++	+++	+++
Feulgen method	—	—	++	++
Sudan Black B (chromated)	—	—	—	+++
Holzinger's technique for fatty acids	+	+	++	++
Fischler's method for fatty acids	0	++	+	+
Copper phthalocyanin	—	+	—	—
Gomori's method (Alkaline phosphatase)	—	—	++	++
Azo dye method (Alkaline phosphatase)	—	—	+	+
Naphthyl acetate method (Esterases)	—	—	—	+
Azo dye method (Acid phosphatase)	—	—	—	—
Lead method (B Glucose-6-phosphatase)	—	—	+++	—

* 0 = Undecided; + = Weak reaction; ++ = Moderate reaction; +++ = Strong reaction; ++++ = Very intense reaction; — = No reaction.

Culture of fertilized eggs

Limulus polyphemus eggs may be cultured in the laboratory following artificial insemination. Eggs were collected and placed in plastic petri dishes. Just enough sea water to form a pool around each batch of eggs was added to prevent desiccation. A 10% spermatozoa suspension was immediately added and the sperm-egg

mixture was allowed to sit for periods of 30 min to 1 hr. The inseminated eggs were then washed three times with sea water and placed in a Percival PT-80 or Sherer RI-LTP Series environmental chamber at 25° C. Throughout the culture period the sea water was changed daily. Inseminated eggs were removed at 5 min, 1 hr and 72 hr, frozen and sectioned at 20 μ , attached to glass microslides or 22 mm sq. cover glasses and immediately fixed in cold (0°-5° C) neutral buffered formalin (Thompson, 1966) for 1 hr. At the end of a 30 min running tap water wash, the inseminated egg sections were stained for mucosubstances with Alcian blue (pH 2.5) nuclear fast red (Humason, 1972). Some egg sections were not chemically fixed following sectioning. Instead they were immediately treated for glucose-6-phosphatase activity (Bancroft, 1967; Pearse, 1968). The glucose-6-phosphatase controls were incubated without the reaction substrate (glucose-6-phosphate) and with complete media and neutral buffered formalin in a 1:1 ratio.

Enzymatic treatment of egg envelopes

To obtain additional information on the nature of the egg envelopes, Cryostat (12 μ) sections of eggs were fixed in neutral buffered 10% formalin (1 hr), washed in running tap water (1 hr) and subsequently treated for 10 min to 8 hr at 37° C with enzyme preparations of malt diastase, hyaluronidase, trypsin, neuraminidase and β -glucuronidase (Thompson, 1966; Spicer and Duvenci, 1964; Lillie, 1947). Malt diastase (Nutritional Biochemicals) was prepared in neutral phosphate buffered saline (1 mg/ml), hyaluronidase (Bovine testes, Type I, Sigma) in 0.1 M acetate buffer (pH 5.0, 0.5 mg/ml), trypsin (4X U.S.P. pancreatin, Nutritional Biochemicals) in M/15 Sorensen's phosphate buffer (pH 7.8, 1 mg/ml), neuraminidase (*Cl. perfringens*, Type V, Sigma) in 0.1 M acetate buffer (at pH 5.0 in final enzyme: buffer conc. of 1:500 and 1:10,000) and β -glucuronidase (Sigma) in 0.004 M phosphate buffer (pH 6.8, 100 units/ml). In addition to the above methods, a number of solutions known to have specific effects on the linkages between proteins (Brown, 1950) and on egg envelopes (Rothschild, 1956; Austin, 1961) were employed in an attempt to obtain more information on how the structural integrity of *Limulus* egg envelopes is maintained. Unfixed Cryostat egg sections (12 μ) were treated for periods up to 24 hr at room temperature or 37° C with 0.2 N HCL (pH 1.2), NaOH (0.2 N, 0.05 N, 0.005 N, pH 12.5), Urea (4%, 6 M), Sodium sulphide (5%), Sodium hypochlorite (10%), Hydrogen peroxide 10%, Ca^{++} free sea water, and Hyamine 1622 in MBL sea water (0.1%).

Amino acid analysis of egg envelopes

Limulus eggs were collected and egg envelopes separated from the yolky components in filtered Instant Ocean synthetic sea water. The separations were accomplished by crushing the eggs with a spatula in a Syracuse watch glass. Both the spatula and watch glass were covered with Parafilm "M" to prevent egg envelopes from adhering to the glass. To remove all traces of yolk, the egg envelopes were washed in three 20-ml vol of filtered sea water in 50-ml plastic beaker with stirring on a Fisher Flexa-Mix magnetic stirrer. Washed eggs were allowed to

dry on glassine powder paper before being prepared for amino acid determination. Each sample prepared for amino acid analysis (3 mg, 6–12 eggs) was placed in a test tube with 1 ml of 6 N-HCl (Moore and Stein, 1960). The test tube was evacuated and sealed under vacuum. The contents were hydrolyzed at 110° C for 22 hr. At the end of the hydrolysis, the HCl was evaporated and replaced with 3 ml of 0.2 N sodium citrate buffer (pH 2.2). The amino acid composition of the sample was determined on a Bechman 120 B Amino Acid Analyzer.

RESULTS

The *Limulus* egg is surrounded by two clearly recognizable layers in sectioned material: the basement lamina and the vitelline envelope. The outer basement lamina is 5 μ thick and transversed by numerous pores. These pores vary in diameter and terminate at the surface of a 35 μ thick layer called the vitelline envelope. At the inner border of the vitelline envelope is a cytochemically identifiable cortical region or zone. This region is approximately 5 μ across in uniseminated eggs. The remainder and bulk of the egg consists of densely packed yolk.

Cryostat section of *Limulus* eggs are easily and quickly prepared for cytochemistry. Sections are attached to cold glass microscope slides or cover glasses without the use of an adhesive. The egg layers adhere to glass strongly and consequently survive many procedures that would ordinarily result in loss of sections.

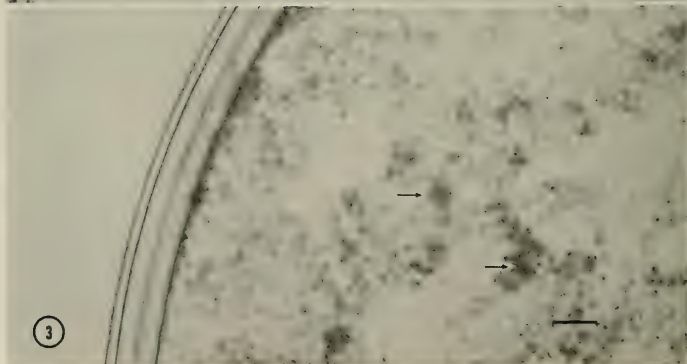
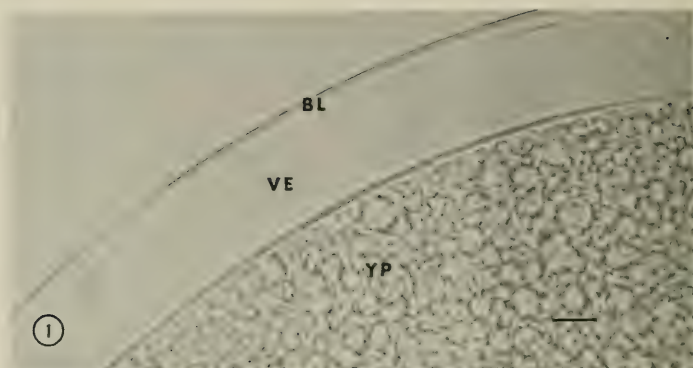
Carbohydrates

The vitelline envelope, cortical region and yolk of *Limulus* eggs are PAS positive. The cortical region and yolk stain red, and the vitelline envelope stains pink, after the PAS reaction. Treatment to block aldehydes with hydroxylamine eliminated the PAS-positive reaction of the egg components.

The yolk and cortical region stain brown following the PAD method for characterizing neutral mucopolysaccharides, periodate-reactive polymers and periodate-unreactive mucosubstances, indicating the presence of periodate-reactive polymers. No staining reaction is observed in the basement lamina following the PAD method.

The presence of 1,2-glycols in the egg is indicated following administration of the CAS and PPS procedures. Whereas all components of the egg give a positive reaction to the PPS method, only the cortical region and yolk give positive reactions with the CAS method. The reaction results in a light pink to light red color with the cortical region and yolk having the same staining characteristics. The vitelline envelope, cortical region and yolk were observed to stain intensely following the PPS procedure, which is in contrast to the weak positive staining reaction of the basement lamina. The most striking observation concerning the PPS procedure is the deposition of dark stain deposits in the canals of the pores, thus demonstrating the vast pore network of the basement lamina.

The Congo red method for glycoprotein demonstrates a strong positive reaction in the cortical region and the yolk. The reaction is much less intense in the basement lamina and vitelline envelope. A positive reaction for sulphated mucosubstances and weakly acidic sulphated mucosubstances is observed only in the cortical region of the egg after staining with Alcian blue at pH 1.0 and pH 2.5 respectively. Similar results are also observed when Alcian blue staining (pH



2.5) is followed by mild methylation to prohibit the staining of nonsulphated mucins. The use of toluidine blue O to demonstrate metachromasia also produces a positive reaction that is restricted to the cortical region. The color reaction following treatment is purple, an indication of γ -metachromasia.

Methylene blue extinction of the basement lamina is between pH 5.3 and 6.7, whereas, that of the vitelline envelope, the cortical region and yolk is between pH 2.6 and pH 4.3. At higher pH values the vitelline envelope displayed a strong affinity for methylene blue (pH 7.6–pH 8.1) while the basement lamina, cortical region and yolk show no increase in affinity for the stain above pH 6.7.

Azure A at pH 1.0, 3.6 and 4.0 produces no staining reactions in the egg.

Proteins and nucleic acids

The entire egg reacts strongly to the mercuric-bromphenol blue method for proteins. The basement lamina stains blue and the vitelline envelope, cortical region and yolk stain blue-purple. While short term deamination is capable of blocking the positive reaction in the basement lamina, only extended deamination of six days is successful in blocking the positive reaction of the vitelline envelope. Even with this extended period of deamination, a weak positive reaction remains in certain yolk bodies. Two other general protein methods, ninhydrin–Schiff and naphthol-yellow S, produce strong positive reactions in the components of the egg. The ninhydrin–Schiff reaction produces a rose-colored staining in the basement lamina and the vitelline envelope with the basement lamina staining darker in color than the vitelline envelope. The yolk, which is not distinguishable from the cortical region, displays a slightly weaker reaction than the vitelline envelope. Hydroxylamine treatment of egg sections prior to employment of the ninhydrin–Schiff method had no effect on their staining characteristics. Deamination with Van Slyke's reagent (Lillie, 1954) blocked the reaction in the yolk and cortical region. Both the basement lamina and the vitelline envelope fail to stain red following this procedure. A weak positive reaction for the basic amino acid arginine appeared in the vitelline envelope and yolk of egg sections with administration of Sekiguchi's method. The basement lamina and vitelline envelope do not stain following the performic acid–Alcian blue technique for disulfides. This is in contrast to the cortical region which displays a much stronger affinity for the stain than the yolk.

A positive Feulgen reaction is observed in the yolk and cortical region. Both regions stain red and cannot be distinguished from each other on the basis of staining intensity. DNase I failed to alter the staining properties after 5 hr at 37° C (sperm smear preparations of *Limulus* spermatozoa were prevented from demonstrating staining of the nucleus after 2 hr at 37° C). Extraction with 1 N HCl at 37° C for 3 hr slightly reduces staining intensity.

FIGURE 1. Control cryostat (12 μ) egg section. Abbreviations are BL, basement lamina; VE, vitelline envelope; and YP, yolk platelets. Scale equals 20 μ .

FIGURE 2. Glucose-6-phosphatase in a cryostat section (12 μ) of an egg. The activity is localized in the cortical region (arrows). Scale equals 35 μ .

FIGURE 3. Glucose-6-phosphatase in a cryostat section (12 μ) of an egg at 72 hr development. Positive reaction product is found in the yolk (arrows) as well as the cortical region (CR). Scale equals 35 μ .

Lipids

The basement lamina and the vitelline envelope are unstained following the Oil red O method for neutral fats, although certain yolk bodies are observed to stain red. The cortical region remains indistinguishable from the yolk in staining characteristics. Similar results are observed following staining with Sudan black B. Controlled chromation and subsequent staining with Sudan black B produced no change in the staining characteristics of the egg yolk. Haematoxylin staining used in conjunction with the Sudan black B procedure stains all yolk bodies as well as the basement lamina and vitelline envelope.

Unsaturated lipids are demonstrated in the vitelline envelope and the yolk with the U-V Schiff method. The cortical region displays light purple staining identical with that of the yolk, while the reaction in the vitelline envelope is somewhat weaker. No staining is observed in controls.

Fischler's method for fatty acids gives a dark blue positive reaction in the vitelline envelope and a lighter reaction in some yolk bodies. No other egg components react. Observations of egg sections following Holczinger's fatty acid technique revealed a greenish-black reaction in all egg components. The strongest reaction is observed in the cortical region and yolk with the staining between these two regions being indistinguishable. Neither methanol-chloroform extraction at 60° C nor 90% ethanol extraction at room temperature has any effect on the staining characteristics. On the other hand, treatment with 1 N HCl or 1 N HCl followed by acetone treatment abolishes staining in all egg components.

A weak positive reaction for lipoproteins is observed in the vitelline envelope following the copper phthalocyanin method. The characteristic blue staining of this procedure is absent from the basement lamina, cortical region and yolk. Methanol-chloroform extraction at 60° C for 24 hr failed to prevent the staining reaction. Controlled chromation at 57 and 60° C over a period of 24 hr produced no change in the staining characteristics of the egg envelopes. The cortical region and yolk elicit a moderate positive reaction.

Enzymes

Two methods for identification of alkaline phosphatases, Gomori's and the Azo dye, give positive results in the cortical region of the egg and in the yolk. With Gomori's method the activity is observed as a brownish color of equal intensity in both the cortical region and the yolk. With the Azo dye method the positive reaction in these two regions is considerably weaker.

Tests to localize acid phosphatases were negative for the entire egg. The yolk gave a positive reaction for non-specific esterases and the cortical region is positive following the procedure for glucose-6-phosphatase.

Cytochemistry of inseminated eggs

Inseminated eggs that were allowed to develop before sectioning are Alcian blue positive in the cortical region. After 4 hr of development the Alcian blue positive region is observed in yolk bodies near the cortical region. At 72 hr the

TABLE II

Effects of physiochemical reagents on Limulus egg sections.

Method or reagent	Effect on egg layers	
	Basement lamina	Vitelline envelope
0.2 N HCl (pH 1.2)	Expansion and increase in size	No change
NaOH		
0.2 N	Expands on contact and dissolves immediately	Dissolves in some instances before basement lamina
0.5 N	Dissolves in approximately 14 min	Dissolves in approximately 14 min
0.005 N	Swelling	No change
Urea		
4%	No change	No change
6 M	Expansion and point separations from the vitelline envelope	No change
Sodium sulphide (5%)	No change	No change
Sodium hypochlorite (10%)	Dissolves	Dissolves
Hydrogen peroxide (10%)	No change	No change
Ca ⁺⁺ free sea H ₂ O	No change	No change
Hyamine 1622 in MBL sea H ₂ O (0.1%)	No change	No change
Trypsin (0.001 g/ml, pH 7.8)—10 min–16 hr	No change	No change

cortical region is no longer Alcian blue positive. A few yolk bodies adjacent to and in the cortical region of 72 hr eggs are Alcian blue positive.

Glucose-6-phosphatase activity, like the positive Alcian blue reaction, is found only in the cortical region of fresh and early developing eggs. At 72 hr the glucose-6-phosphatase activity is observed in the yolk and is indistinguishable in intensity from that of the cortical region.

Enzymatic treatment of uninseminated egg sections

The enzymatic treatments had no effect on the staining characteristics of the egg sections in all cases except one when compared with untreated sections. β -Glucuronidase treatment for a period of 8 hr was the only enzymatic treatment to produce a decrease in staining intensity which is observed in the vitelline envelope, cortical region and yolk of uninseminated egg sections.

Treatments with trypsin, malt diastase, or hyaluronidase causes most of the yolk to become suspended in the enzyme solutions. Coating sections with celloidin (Thompson, 1966) affords a small degree of protection if the microslides are handled gently. The enzyme solutions do not digest either the basement lamina or the vitelline envelope.

Physiochemical treatment of egg sections

A number of reagents were employed to determine the types of bonds responsible for maintaining the structural integrity of the *Limulus* egg envelopes. The

TABLE III

Amino acid composition of uninseminated and inseminated (72 hr) Limulus egg envelopes.*

Amino acid	Micromoles/uninseminated egg	Micromoles/inseminated egg	Increase at 72 hr level (%)
Lysine	0.097	0.188	93.8
Histidine	0.028	0.066	135.7
Ammonia	0.119	0.221	85.7
Arginine	0.031	0.058	87.0
Aspartic acid	0.126	0.200	58.7
Threonine	0.295	0.121	-58.9
Serine	0.090	0.147	63.3
Glutamic acid	0.114	0.203	78.0
Proline	0.087	0.156	79.3
Glycine	0.081	0.148	82.7
Alanine	0.044	0.079	79.5
Half Cystine		0.079	+ - -
Valine	0.100	0.186	86.0
Methionine	0.011	0.018	63.6
Isoleucine	0.092	0.168	82.6
Leucine	0.106	0.184	73.5
Tyrosine	0.129	0.178	37.9
Phenylalanine	0.058	0.090	55.1

* Mean of 3 determinations.

† Not present in uninseminated egg investments.

results of these treatments for periods up to 24 hr at room temperature are recorded in Table II.

Amino acid analysis of isolated uninseminated egg envelopes

Sixteen amino acids are found in the egg envelopes. Amino acids containing sulfur as a sulfhydryl group (cysteine) and sulfur in a thioether linkage (Methionine) are absent or in low concentrations. Also absent are the amide forms of aspartic and glutamic acid. The amino acid composition of *Limulus* egg envelopes has been tabulated in Table III.

Amino acid analysis of isolated 72 hr egg envelopes

Seventeen amino acids are found in the egg envelopes at 72 hr of development. The amino acid cystine, which is not present in uninseminated egg envelopes, is present in 72 hr egg envelopes. Each amino acid found in the uninseminated egg envelopes demonstrated a significant percentage of increase over the amount found in 72 hr egg envelopes (Table III). The average amount of increase for all amino acids found in uninseminated egg envelopes as compared with 72 hr egg envelopes is 71.5%.

DISCUSSION

The PAS procedure is a histochemical technique that stains tissue components containing 1,2-glycols. The intensity of the reaction is dependent upon the con-

centration of the adjacent glycol or aminohydroxyl groupings in the tissue (Thompson, 1966). Observations that the vitelline envelope, cortical region and yolk are strongly PAS positive is indicative of the presence of a high concentration of nondiffusible 1,2-glycol groups in these components of the *Limulus* egg. Such findings are further substantiated by the ability of an aldehyde blocking reagent (hydroxylone) to eliminate completely the PAS positive reaction of the vitelline envelope, cortical region and yolk. Additional evidence supporting the presence of 1,2-glycol groups in the *Limulus* egg comes from positive results following the CAS and PPS procedures, both of which indicate the presence of 1,2-glycol groups.

The presence of sulfated mucopolysaccharides is revealed in the egg cortex following staining with Alcian blue. In support of this finding is the observation that mild methylation has no effect on the alcianophilia of this region. The fact that the cortical region of the *Limulus* egg is azure A negative is also of interest. Spicer (1963) found some rodent mucopolysaccharides failed to display an affinity for azure A, but were positive for Alcian blue. An analysis of these mucopolysaccharides demonstrated that many of the Alcian blue positive-azure A negative mucopolysaccharides contained sulfuric acid groups.

Neuraminidase treatment and subsequent failure to eliminate alcianophilia in the *Limulus* egg cortex suggests sialic acid is not responsible for the acidic nature of the substances within this region. In addition, hyaluronidase treatment failed to demonstrate any effect on the alcianophilia of the cortical region of the *Limulus* egg, indicating uronic acids cannot be responsible for the observed staining characteristics. The inability to localize either sialic acids or uronic acid, two important constituents of glycosaminoglycans, indicates the protein-polysaccharide substances in the cortical region of the *Limulus* egg are glycoproteins in nature. Observations that the cortical region of *Limulus* eggs contain sulfated mucosubstances and glycoproteins is of interest since cytochemical studies have demonstrated these substances in cortical granules of other species (Monne and Harde, 1951; Szollosi, 1962; Schuel, Wilson, Bressler, Kelley and Wilson, 1972).

The Alcian blue positive reaction observed in uninseminated egg sections is not present in sections of inseminated and developing eggs beyond 72 hr of development. The dissipation of this cytochemically defined region in inseminated eggs is probably analogous to the dissipation of cortical granule material following rupture of the cortical granules in other species (Austin, 1965, 1968). Since there is no change in the location of the material in the cortical region of the *Limulus* egg immediately following insemination, the macromolecules of this region are probably not involved in the production of a barrier against polyspermy. This notion is further supported by the observation that this chemically defined region appears in both uninseminated and inseminated eggs. Instead, the macromolecules of this region most likely participate in the formation of the embryonic egg envelope of the *Limulus* embryo. This is suggested from observations that the dissipation of the sulphated mucosubstances of the cortical region at 72 hr of development coincides with the appearance of half cystine in amino acid hydrolysates of the original egg envelopes; the layers that give rise to the embryonic egg envelope.

Considerable protein is found in the basement lamina, vitelline envelope, cortical region and yolk of the *Limulus* egg as is demonstrated by the staining affinity of

these components following mercuric-bromphenol blue, naphthol-yellow S and the ninhydrin-Schiff methods. Treatment of egg sections with Van Slyke's reagent, a deaminating reagent, progressively blocks the staining of *Limulus* egg sections subsequently stained with mercuric-bromphenol blue. Since this reagent blocks most rapidly α -amine groups and staining with mercuric-bromphenol blue follows the Beer-Lambert laws (Mazia, Brewer and Alfert, 1953), it can be concluded that the basement lamina of the *Limulus* egg contains the least number of α -amine groups in the egg since this region is the first part of the egg that fails to stain with mercuric-bromphenol blue following deamination.

The localization of DNA in the cortical region and the yolk of *Limulus* egg sections was revealed following the Feulgen reaction. The extreme resistance of this DNA to extractive methods is indicated following DNase I treatment and $1 \times \text{HCl}$ extraction. Even though DNase I was found to prevent Feulgen staining in *Limulus* sperm nuclei, it had no effect on the cortical region and yolk of *Limulus* egg sections. Since the Feulgen method is specific for DNA, it is concluded the DNase-I-sensitive sites in *Limulus* sperm nuclei are more accessible than those of the *Limulus* egg cortical region and yolk.

DNA is known to be associated with the cytoplasm of sea urchin eggs (Baltus, Quertier, Ficq and Brachet, 1965) and with mitochondria and yolk platelets in amphibian eggs (Brachet, 1967). The physical and chemical properties of cytoplasmic DNA are similar to nuclear DNA in sea urchin and *Xenopus* eggs (Brachet and Malpoix, 1971). The present study demonstrates a large amount of extra-nuclear DNA in the *Limulus* egg is also associated with yolk (yolk bodies). The role of yolk DNA in the *Limulus* egg at present is speculative. One possible role could involve participation in yolk body breakdown during embryogenesis as is suggested in studies on amphibian eggs (Brachet and Malpoix, 1971). Still another function of yolk DNA could be to supply nucleotides for nuclear DNA replication during development of the *Limulus* embryo.

The various classes of lipids demonstrated in *Limulus* egg sections are primarily localized in the yolk. The demonstration of neutral fats following the Oil red O method reveals that not all the yolk bodies constituting the yolk are colored by this procedure. Neutral fats appear to be restricted to certain yolk bodies. This is also true of phospholipids demonstrated with Sudan black B staining in conjunction with controlled chromation and hematoxylin staining. Extraction techniques were used with many of the lipid staining procedures. The general outcome was the same, they failed to prevent staining of the lipids. The results demonstrate the need for caution in accepting the effects of extractive techniques as being similar to effects obtained *in vitro*. Interpretations based on the effects of extractive techniques should be held in reserve until parallel chemical studies can be conducted on each technique (Pearse, 1968). The failure of lipid extraction techniques to affect the staining characteristics of *Limulus* egg sections is not surprising since extraction and digestion techniques for carbohydrates and proteins also have no effect on staining characteristics. The macromolecules of the *Limulus* egg are apparently well protected from the degradative effect of a number of enzymes and extractive techniques. This might be the key to the survival of this primitive organism. Even early investigators (Kingsley, 1892) have noted the hardness of the *Limulus* egg.

Of the various reagents tested on *Limulus* egg sections, only those treatments specific for disrupting electrovalent bonds (HCl, NaOH, urea), covalent bonds other than those formed by disulphide linkages (sodium hypochlorite), and pepsin had any noticeable effect on the egg envelopes.

Interpretation of results demonstrating involvement of electrovalent linkages in maintaining the structural integrity of the *Limulus* egg envelope presents some difficulty when covalent linkages are also involved. To break covalent linkages, specifically the -S-S- bond, a sodium sulphite solution may be employed. The negative results obtained from this procedure demonstrated the absence of -S-S- linkages in the *Limulus* egg envelopes. Apparently in these egg envelopes, unlike those of some species (Gusseck and Hedrick, 1971), disulphide bonds do not play an integral part in maintaining structural integrity. On the other hand, covalent linkages other than -S-S- linkages are important in holding the protein chains together in both the basement lamina and vitelline envelope of the *Limulus* egg since sodium hypochlorite treatment dissolves these two layers.

Sixteen amino acids were identified from the acid hydrolysate of uniseminated *Limulus* egg envelopes and 17 residues were identified from similar hydrolysates obtained from the envelopes of inseminated and developing eggs. The amino acid composition of uniseminated egg envelopes suggests the protein or group of proteins in the egg envelopes are primarily acidic. The most abundant amino acids are tyrosine, aspartic acid and glutamic acid. Of particular interest is the relatively large amount of tyrosine found in *Limulus* egg envelopes. Relatively large amounts of tyrosine are also found in the egg shells of some insects, namely the fruit fly (Wilson, 1960) and the silkworm (Tomita, 1921). It is well established that polyphenols and their quinone derivatives play an important role in the darkening and hardening of the insect cuticle. The polyphenols are derived from tyrosine which is oxidized by tyrosinase (Richards, 1951). In the insect cuticle this enzyme system is widely distributed so that hardening and darkening of the cuticle is controlled by the distribution of the substrate, tyrosine. From the amino acid data on *Limulus* egg envelopes it could be postulated that the observed toughness of these egg envelopes is due to a similar mechanism involving tyrosine, but that darkening of the envelopes is not as pronounced as in the insect cuticle.

Of equal interest is the absence of the amino acid cysteine in uniseminated *Limulus* egg envelopes. Recently, Gusseck and Hedrick (1971) postulated on the importance of sulfhydryl groups in maintaining the integrity of egg envelopes (jelly) in amphibians and in the process of fertilization (membrane fusion). According to these authors, the structural integrity of jelly envelopes of *Xenopus laevis* as well as egg envelopes of other species displaying susceptibility to solubilization by reagents which break disulfide bonds are maintained by disulphide bonds. In addition, sperm-egg membrane fusion is also thought to be a direct result of the formation and interchange of sulfhydryl-disulfide bonds. Neither hypothesis involving sulfhydryl-disulfide bond formation would appear to be applicable to gametes of *Limulus*. Physiochemical tests reveal disulfide bond breaking reagents have no solubilizing effect on egg envelopes of *Limulus* and amino acid analysis reveals cysteine to be absent from *Limulus* egg envelopes.

Amino acid analysis of egg envelopes from inseminated and developing *Limulus*

eggs (72 hr development) demonstrates the presence of half-cysteine. In addition, all other amino acids found in uniseminated egg envelopes show significant increases in amounts in egg envelopes from inseminated and developing eggs. The presence of half-cysteine in *Limulus* egg envelopes makes these envelopes identical in amino acid composition with the egg membranes obtained from blastula- and gastrula-stage embryos of *Bufo vulgaris* (Uchiyama, Ishihara and Ishida, 1971), the egg shells of *Acheta domesticus* (Furneaux, 1970) and with the chorions of *Fundulus heteroclitus* (Kaighn, 1964) and *Salmo solar* (Young and Smith, 1956). Egg envelopes of these species, like those of *Limulus*, also contain relatively large amounts of acidic amino acid residues.

The appearance of half-cysteine and the incorporation or increase in amount of amino acids in the egg envelopes at 72 hr following insemination probably have significance in events that will take place during subsequent embryonic development. The production or synthesis of the embryonic envelope which surrounds the embryo at the imbibition-swelling stage is one possible explanation for the tremendous increase in amount of amino acids present in the egg envelopes. The amino acids for the embryonic envelope, which develops inside the original egg envelopes, may begin accumulation as early as 72 hr following insemination.

SUMMARY

Cytochemical studies on uniseminated mature eggs of *Limulus* demonstrate the presence of carbohydrates, lipids and proteins in the egg envelopes and yolk. The vitelline envelope, cortical region and yolk are rich in 1,2-glycols, with the vitelline envelope containing fewer reactive 1,2-glycol groups than other components of the egg. Neutral mucopolysaccharides are found in the cortical region and yolk, but only the cortical region of the eggs demonstrate the presence of sulfated mucosubstances (which are in part glycoprotein in nature) and glucose-6-phosphatase. Protein is evident in all egg components. Biochemical analysis demonstrate the protein in the egg envelopes of uniseminated eggs is composed of sixteen amino acids while that of developing eggs contain seventeen amino acid residues. Electrovalent linkages and non-S-S- covalent linkages between protein chains are shown to be instrumental in maintaining the structural integrity of *Limulus* egg envelopes. Neutral lipids, unsaturated lipids, phospholipids and fatty acids are demonstrated in yolk bodies and lipoproteins, unsaturated lipids and fatty acids constitute part of the egg envelopes. DNA is concentrated in the cortical region and the yolk bodies.

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AEROBIC AND ANAEROBIC METABOLISM DURING ACTIVITY AND HYPOXIA IN TWO SPECIES OF INTERTIDAL CRABS¹

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Intertidal crabs are active under many environmental conditions. They must supply energy for activity in both air and water, and over a range of microhabitat temperatures. This energy may be produced aerobically, or anaerobically without the immediate use of oxygen. Anaerobiosis is particularly important when environmental oxygen is low or absent, or when respiratory and circulatory systems cannot supply oxygen at a rate sufficient to meet metabolic demands.

It is known that during burst activity or diving, when oxygen supply to muscles is limited, vertebrates rely almost exclusively on the Emben-Meyerhof pathway leading to lactate production for energy. The ectothermal vertebrate classes (fish, amphibians and reptiles) are particularly reliant on anaerobically-produced energy for activity because of strong respiratory and cardiovascular limitations on their aerobic metabolic rate (Bennett and Licht, 1973). Such burst activity produces high body lactate levels and quickly leads to fatigue.

Intertidal crabs are also an active, ectothermal group, and should also be reliant on anaerobiosis to support vigorous activity. The possibility that glycolysis is a major route of energy production during activity is supported by the finding that crustacean muscle converts pyruvate to lactate during activity (Dendinger and Schaltzein, 1973; Phillips, McKinney, Hird and Macmillan, 1977). The qualitative importance of anaerobiosis might change with environmental conditions, since temperature and respiratory media strongly influence the availability of O₂ for aerobic metabolism.

The stress of low levels of environmental oxygen has been met by other invertebrate groups with a diversity of respiratory and biochemical mechanisms (see Newell, 1973 for review). Metabolic rate may fall during the hypoxic period, or may be maintained by a combination of aerobic and anaerobic mechanisms, including increasing ventilation frequency, utilizing stored oxygen (Mangum and Van Winkle, 1973), and producing energy via anaerobic biochemical pathways. The most quantitatively significant end products are thought to be succinate, volatile fatty acids (Saz, 1971), and alanine (de Zwaan and Zandee, 1972, a, b). Lactic acid is a minor end product in some species (Hammen, 1975). However, crustaceans appear to differ from other invertebrates in that lactate is a significant end product during hypoxia (Teal and Carey, 1967), which is also the situation in vertebrates (Burton and Spehar, 1971; D'Eon, Boutillier and Toews, 1978). Thus we might ask whether some intertidal crab species rely on glycolysis for energy production when faced with hypoxic environmental conditions. Further, since the

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selection pressures to utilize anaerobiosis during hypoxia or during activity are often quite distinct, we might ask if the anaerobic responses to activity and hypoxia are independent.

MATERIALS AND METHODS

This study examines the aerobic and anaerobic response to activity and hypoxia of *Pachygrapsus crassipes*, the lined shore crab, which lives exclusively in oxygen-rich intertidal environments (Hiatt, 1948). To determine whether these crabs are reliant on anaerobiosis to support vigorous activity, the rate of oxygen consumption (V_{O_2}) and lactate accumulation was measured to estimate aerobic and anaerobic contributions to energy production, respectively, in crabs at rest and active, in air and water. To determine whether *Pachygrapsus crassipes* relied on glycolysis during environmental hypoxia the oxygen consumption and lactate accumulation of crabs submerged in hypoxic sea water was measured. To determine whether the anaerobic responses to activity and hypoxia are independent, the metabolic responses of *Pachygrapsus crassipes* were contrasted with those of *Carcinus maenas*, the green crab, a species which encounters hypoxic conditions in mud and stagnant water, and air exposure which may lead to hypoxic haemolymph (Taylor, Butler and Sherlock, 1973).

Specimens of *P. crassipes* were collected in Corona del Mar, Orange County, California; collection was permitted by California sport fishing licenses. The crabs were held individually at 15° C in 1–2 cm filtered sea water. They were used within two weeks of collection and remained unfed at least three days before experiments. Specimens of *Carcinus maenas* were supplied by the Woods Hole Marine Biological Laboratory Experimental Invertebrate Zoology course, Barnstable County, Massachusetts. They were held at 22.5° C in tanks with flowing filtered sea water and fed marine invertebrates every few days.

To estimate the quantitative importance of anaerobiosis the L-lactate concentrations of crabs were measured. D-lactate concentrations were not measured because all mandibulate arthropods, excepting the myriapods, possess only the L-stereoisomer of lactate dehydrogenase (Long, 1976). When only a single determination was required whole body levels were the index of the total lactate production; when serial determinations were required, haemolymph concentrations were used. These reflect relative changes in the lactate concentration of an individual crab.

The effect of burst activity on haemolymph lactate levels was measured in both species. Individual specimens of *Carcinus maenas* were prodded to stimulate locomotion in air and in 6 cm aerated sea water at 22.5° C. After 15 min of stimulation a haemolymph sample was collected from a walking leg, and mixed with cold 0.6 N perchloric acid. The sample was kept on ice 10–20 min, centrifuged 10 min and filtered. Supernatant L-lactate concentration was assayed according to the method of Gutman and Wahlefeld (1974). Haemolymph from specimens of *P. crassipes* was collected as above. Prior to the initial haemolymph sample crabs were kept isolated in about 1 cm sea water for 15–20 hr. Activity was then induced for 5 min in air at 15° C. Haemolymph samples were collected

immediately following activity. Haemolymph samples were also collected from a control group not stimulated to run.

Since such burst activity caused lactate production and rapidly led to fatigue, the attempt was made to see if fatigue was correlated with maximum lactate concentration. The criterion for fatigue was a loss of righting response (lasting 15 sec while the crab was constantly prodded). Whole body lactate levels of specimens of *P. crassipes* were measured at rest, at loss of righting response, and after continued forced activity. Crabs that had been held at the experimental temperature overnight were stimulated to locomotion in air and 6 cm aerated sea water at 15 and 25° C. Immediately following a loss of righting response a crab was homogenized with ten times its weight of cold 0.6 N perchloric acid. Lactate concentrations in the supernatant were determined with a Boehringer-Mannheim lactic acid analysis kit. Whole body lactate concentrations of crabs held at 15 or 25° C undisturbed overnight and those stimulated to activity for 5 min beyond fatigue were also determined.

To determine the aerobic component of energy production during activity, the aerial $\dot{V}_{O_2}$ of specimens of *P. crassipes* was measured. Crabs were kept in individual plexiglass chambers at 15° C for 15–20 hr prior to measurements, with 1 cm sea water and constant air flow. Water vapor and CO_2 were removed from incurrent and excurrent air with Drierite (anhydrous $CaSO_4$) and Ascarite (Na-hydrate asbestos), respectively. The partial pressure of oxygen (P_{O_2}) was determined on a dry, CO_2 -free sample by a Beckman E_2 oxygen analyzer. Partial pressure of oxygen of the incurrent air flow was analyzed before and after each sample.

Resting $\dot{V}_{O_2}$ was calculated from the average P_{O_2} of three consecutive samples of excurrent air, taken 15 min apart, and standardized to STP. The rate of oxygen consumption during activity was determined within 3 hr following determinations of resting $\dot{V}_{O_2}$. The chamber was closed, and an initial sample of chamber air was dried and the CO_2 removed for determination of P_{O_2} and was replaced by dry, CO_2 -free room air. The crab was then stimulated to activity for 5 min by prodding it with a small stir bar inside the chamber, manipulated by a large stir bar beneath the chamber. This activity was assumed to be maximal since each crab lost its righting response for at least 15 seconds during the activity period. A final 20 ml sample of chamber air was removed for analysis of fractional O_2 content. Oxygen uptake was calculated according to the formula of Bennett and Gleeson (1976). All reported values are corrected for STP and include compensation for CO_2 removal (Depocas and Hart, 1957) and relative humidity.

The relationship between temperature and rates of aerobic and anaerobic metabolism was also evaluated in the preceding experiments. To quantify the effect of temperature on burst activity the running speed of individuals of *Pachygrapsus crassipes* down a cloth-covered track was measured using two photocells connected to an electronic timer. Crabs were held at the experimental temperature (15 or 25° C) overnight prior to the experiment. Burst speed was measured over a distance of 200 cm. The speed of each crab was measured three times, with a 5 min period between each running trial.

The aerobic and anaerobic response to hypoxia was examined in both crab species. The resting $\dot{V}_{O_2}$ of specimens of *Carcinus maenas* submerged in 22.5° C

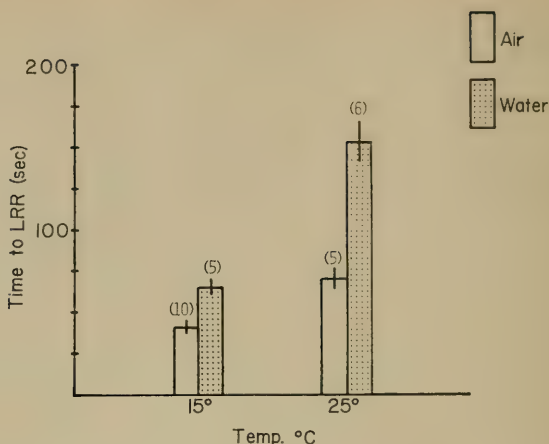


FIGURE 1. Duration of activity (in sec) of specimens of *Pachygrapsus crassipes* until loss of righting response. Submerged crabs remained vigorously active longer in water than in air, and in both media a loss of righting response occurred more quickly at 15° C than at 25° C.

sea water was determined from the rate at which the crabs reduced the P_{O_2} (Mangum and Van Winkle, 1973). The $\dot{V}_{O_2}$ of a single submerged crab in a sealed dark chamber was calculated from the P_{O_2} of water samples analyzed with a physiological blood gas analyzer. Oxygen uptake by specimens of *Carcinus maenas* stopped (aerobic shutdown) between water P_{O_2} of 15 and 25 torr. Haemolymph samples for lactate analysis were collected from crabs held in air-saturated water for 2 hr, crabs at aerobic shutdown, and from crabs held in hypoxic water 3 hr after aerobic shutdown.

The $\dot{V}_{O_2}$ of specimens of *P. crassipes* submerged in 15° C sea water was continuously monitored with a Beckman Fieldlab oxygen analyzer. When the crabs had reduced water P_{O_2} to 10 torr they were quickly homogenized for determination of whole body lactate.

Unless otherwise indicated, all values are reported as the mean $\pm$ 1 standard error. Sample size is given in parentheses. In all statistical tests, differences are considered significant when $P \leq 0.05$. A Newman-Keuls multiple range test (Zar, 1974) was used to test for differences between mean $\dot{V}_{O_2}$ at successively lower P_{O_2} . A two by four factorial ANOVA (Winer, 1962) was used to test for differences between mean whole body lactate concentration after activity at different temperatures and in different respiratory media. A two-factor ANOVA for repeated measurements on the same subject (Winer, 1962) was used to test for differences between mean lactate concentrations of serial haemolymph samples and between mean running speeds during consecutive trials. In all other experiments a two-tailed Student's *t*-test was used to determine differences between means.

RESULTS

The results indicate that intertidal crabs rely on anaerobiosis to support vigorous activity, that some intertidal crab species rely on glycolysis for energy production during environmental hypoxia, and that the abilities to utilize anaerobiosis during activity or during hypoxia are independent.

Crabs of both species appear to be capable of only short bursts of vigorous activity, but the duration of this activity was affected by the media and the ambient temperature. Half the specimens of *Carcinus maenas* active under water did not exhibit a loss of righting response after 15 minutes of activity, but all specimens of *Carcinus* exercised in air did fatigue. A loss of righting response of the crabs which fatigued while submerged occurred after 476 ± 110 sec ($N = 4$), but after only 189 ± 40 sec ($N = 8$) for crabs active in air. Specimens of *Pachygrapsus crassipes* remained vigorously active longer in water than in air, and in both media a loss of righting response occurred more quickly at 15°C than at 25°C (Fig. 1).

Initial burst running speed of specimens of *P. crassipes* was independent of temperature at 15 and 25°C ; however, in subsequent running trials the colder crabs ran slower than the warmer crabs (Fig. 2). After the initial burst of running, crabs were slow to right themselves if overturned, indicating that fatigue probably caused the observed decrease in speed.

Burst activity in these crabs results in lactic acid accumulation. The rate of lactate formation was not clearly dependent on temperature or on media in *Pachy-*

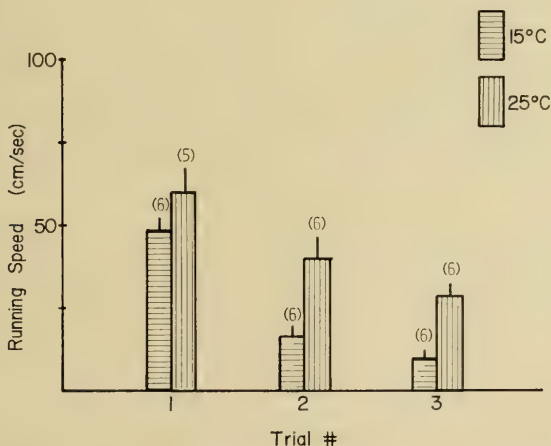


FIGURE 2. Running speed (in cm/sec) of *Pachygrapsus crassipes* at two temperatures. Initial burst running speed (Trial 1) was independent of temperature, but in subsequent running trials (2 and 3) the colder crabs did not run as fast as the warmer ones.

TABLE I

Whole body lactic acid concentrations (mg lactate/g) of specimens of *Pachygrapsus crassipes* held at either 15 or 25° C. Most crabs kept submerged in aerated water at 25° C died; therefore, that condition is omitted. Vigorous activity in either media results in high lactate concentration, and crabs forced to be active beyond fatigue show no further increase in lactate levels.

Temperature	Media	Rest	Activity to a loss of righting response	5 min activity
15° C	Air	0.350 ± 0.088 (4)	0.605 ± 0.061 (9)	0.611 ± 0.072 (6)
	Water	0.327 ± 0.011 (11)	0.725 ± 0.073 (12)	—
25° C	Air	0.427 ± 0.047 (6)	0.586 ± 0.072 (6)	0.498 ± 0.047 (6)
	Water	—	0.808 ± 0.084 (6)	—

grapsus crassipes. *P. crassipes* specimens exercised to fatigue at 15 or 25° C had similar lactate concentrations despite the greater duration of activity at 25° C (Table I). Lactate concentrations of resting and post-active specimens of *P. crassipes* were about the same in air and water. However, a crab exercised in water at 25° C until it lost its righting response had a lactate concentration twice as high as that of a crab exercised in air. Crabs stimulated to 5 min activity in air at these temperatures showed no further increase in lactate over concentrations at the loss of righting response (after 0.5 to 3 min activity), suggesting anaerobic capacity was reached in the initial burst of activity.

Haemolymph lactate concentrations followed the pattern of whole body concentrations. Haemolymph lactate doubled (from 0.409 ± 0.141 to 0.954 ± 0.155 mg/ml) in specimens of *Pachygrapsus* forced to be active for 5 min in air at 15° C, as did whole body concentrations (Table I). Unstimulated crabs showed no increase in haemolymph lactate over a similar 5 min period. Haemolymph lactate concentrations of resting animals were 1.3–2.2 times whole body concentration, making it a possible predictor of whole body concentration.

Burst activity in *Carcinus maenas* caused a rise in haemolymph lactate similar to that observed in *P. crassipes*. Resting haemolymph lactate levels were the same in submerged and air-exposed crabs (Table II). Concentrations increased

TABLE II

Haemolymph lactic acid concentrations (mg/ml) of specimens of *Carcinus maenas* at rest, after 15 min forced activity in air or water at 22.5° C, and during hypoxic exposure in 22.5° C water. A single sample was taken from each crab. Activity resulted in high lactate concentrations. Running in air caused greater increases than running while submerged. Hypoxic conditions also resulted in high lactate levels, and when crabs stopped aerobic metabolism at low P_{O_2} 's lactate concentrations increased ten-fold over initial concentrations.

Rest, air	0.22 ± 0.11 (4)
Rest, water	0.25 ± 0.07 (8)
15 min active, air	1.19 ± 0.13 (8)
15 min active, water	0.64 ± 0.14 (8)
Aerobic shutdown	0.94 ± 0.20 (6)
3 hr after shutdown	2.51 ± 0.69 (5)

after 15 min forced activity at 22.5° C, and activity in air resulted in lactate levels twice as high as activity in water.

The aerobic contribution to energy production during activity was measured in specimens of *P. crassipes* at 15 and 25° C. Although resting $\dot{V}_{O_2}$ was higher at 25° C than 15° C (0.145 ± 0.024 vs. 0.034 ± 0.003 ml O_2 g per hr), the aerobic contribution during activity was the same (0.192 ± 0.021 vs. 0.197 ± 0.021 ml O_2 g per hr). Resting $\dot{V}_{O_2}$ at 25° C may be overestimated due to spontaneous activity.

Respiratory media affected $\dot{V}_{O_2}$ in specimens of *P. crassipes*. The resting $\dot{V}_{O_2}$ was higher in aerated water than in air (0.052 ± 0.009 ml O_2 g per hr, $N = 7$, mean wt = 15.7 g vs. 0.034 ± 0.003 ml O_2 g per hr, $N = 17$, mean wt = 18.5 g).

The respiratory and biochemical response of submerged crabs to progressive hypoxia differed between the two species. Specimens of *P. crassipes* at 15° C ($N = 7$, mean wt = 15.7 g) decreased their rate of aerobic respiration as P_{O_2} declined, but they continued oxygen uptake at $P_{O_2} = 10$ torr (Fig. 3). Whole body lactate concentrations of specimens of *P. crassipes* were greater in water at $P_{O_2} \leq 10$ torr than the whole body lactate concentration of crabs in water saturated with air ($P_{O_2} = 121\text{--}160$ torr) (0.558 ± 0.036 mg/g, $N = 8$ vs. 0.327 ± 0.001 mg/g, $N = 11$).

In contrast to the response of *P. crassipes* to declining levels of oxygen, *Carcinus* specimens maintained resting $\dot{V}_{O_2}$ down to $P_{O_2} = 50\text{--}60$ torr (Fig. 4). The rate of oxygen uptake then decreased to ca. 1/3, and $\dot{V}_{O_2}$ stopped entirely (aerobic

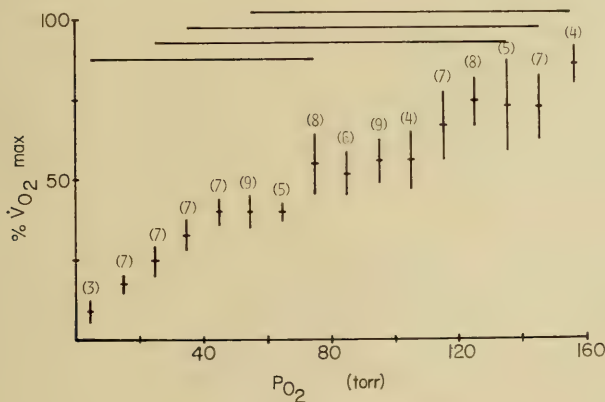


FIGURE 3. The rate of oxygen consumption of *Pachygrapsus crassipes* as a function of water P_{O_2} at 15° C. Crabs depleted oxygen in sealed vessels. Values expressed as the mean fraction of the highest observed $\dot{V}_{O_2}$ of undisturbed crabs (ml O_2 g per hr). Horizontal lines connect means not significantly different at $P = 0.05$ according to the Newman-Keuls multiple range test. Crabs decreased their rate of oxygen uptake as P_{O_2} declined, but they continued to consume oxygen at $P_{O_2} = 10$ torr.

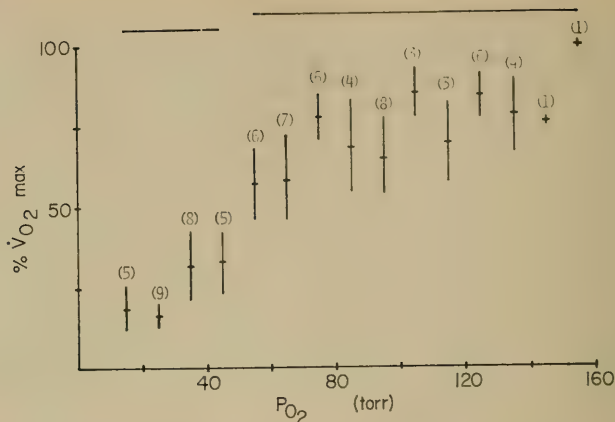


FIGURE 4. The rate of oxygen consumption of *Carcinus maenas* as a function of water P_{O_2} at 22.5° C. Crabs depleted oxygen in sealed vessels. Values expressed as the mean fraction of the highest observed $\dot{V}O_2$ (ml O_2 /g per hr) of undisturbed crabs. Horizontal lines connect means not significantly different at $P=0.05$ according to the Newman-Keuls multiple range test. Crabs maintained resting $\dot{V}O_2$ down to $P_{O_2}=50$ -60 torr, then $\dot{V}O_2$ decreased to ca. 1/3 the resting rate. Oxygen uptake stopped entirely between 10 and 25 torr P_{O_2} .

shutdown) between 10 and 25 torr. Haemolymph lactate concentrations of *Carcinus maenas* increased during progressive hypoxia from 0.25 ± 0.07 mg/ml ($N=8$) to 0.94 ± 0.20 mg/ml ($N=6$) at aerobic shutdown. Crabs maintained at shutdown for 3 hr had haemolymph lactate concentrations that were 10 times higher than those in aerated water (2.511 ± 0.69 mg/ml, $N=5$), but they remained responsive and lived for at least one day upon return to aerated water.

DISCUSSION

The ability of an animal to respond to energetic demands incorporates both aerobic and anaerobic energy sources. The relative utilization of these two routes of energy production in a given species is dependent on the metabolic capacities of the animal, oxygen availability in its environment, and the intensity of activity. The dependence of *Pachygrapsus crassipes* on anaerobiosis for energy was estimated using rates of oxygen consumption and lactate production as indices of aerobic and anaerobic metabolism, respectively. Relative ATP yields were estimated from rates of oxygen consumption and lactate production using the values given by Bennett and Licht (1972). One mg lactate formed = 16.7 μ -moles ATP, assuming that glycogen is the only substrate catabolized. One ml O_2 (STP) consumed = 290 μ -moles ATP, assuming a P/O ratio of three.

The aerobic/anaerobic energy balance of a specimen of *P. crassipes* vigorously active for 5 min was estimated. It was assumed that $\dot{V}O_2$ was constant during that

period and that all lactate was formed during the first min of activity. The energy balance during the first min of burst activity at 15° C is:

$$\begin{aligned}3.8 \mu\text{l O}_2/\text{g} &= 1.1 \mu\text{moles ATP/g} \\0.261 \text{ mg lactate/g} &= 4.4 \mu\text{moles ATP/g} \\ \text{total} &= 5.6 \mu\text{moles ATP/g}\end{aligned}$$

and at 25° C:

$$\begin{aligned}3.3 \mu\text{l O}_2/\text{g} &= 1.0 \mu\text{moles ATP/g} \\0.148 \text{ mg lactate/g} &= 2.5 \mu\text{moles ATP/g} \\ \text{total} &= 3.5 \mu\text{moles ATP/g}\end{aligned}$$

These calculations show the initial burst of running is primarily anaerobic at both 15 and 25° C, within the range of temperatures normally encountered by this species in the collection area. Initial burst running speed is also temperature independent. This independence of initial running speed may be the result of thermal independence of lactate production. Such temperature independence of anaerobiosis has been demonstrated in lizards (Bennett and Licht, 1972).

Since individuals of *Pachygrapsus crassipes* are primarily anaerobic during burst activity, vigorous activity only occurs in short bursts and quickly leads to fatigue. This permits crabs to quickly attain high speeds for short distances independent of ambient temperature, but limits their ability to maintain activity.

Intertidal crabs are active in both water and air, and these media have very different properties affecting locomotion and respiration. For example, water provides more body support than air, and gills adapted for oxygen uptake in both water and air are probably not equally efficient in both media (Gray, 1957). Directed ventilation is more difficult with air, and gill platelets supported by water tend to collapse in air. Although these factors must be balanced against the much greater concentration of oxygen in air than in water, one might predict that it is more difficult for an intertidal crab to increase $\dot{V}_{\text{O}_2}$ when exposed to air than when it is submerged.

The results support this prediction. Submerged specimens of both *P. crassipes* and *Carcinus maenas* were able to sustain vigorous activity longer than those exposed to air. This difference was more pronounced in *Carcinus maenas* specimens. Also, *Carcinus maenas* specimens had a greater post-active accumulation of lactate in haemolymph after activity in water, whereas *P. crassipes* individuals showed little difference in lactate concentration between activity in air or water at 15° C. In fact, lactate levels after activity at 25° C were greater in submerged than in air-exposed crabs.

The respiratory responses of crabs to submersion and air exposure are correlated with the environmental constraints on respiration each species faces. *P. crassipes* is strictly intertidal and constantly moves between air and water. *Carcinus maenas* stays submerged throughout the tidal cycle and migrates offshore for the winter (Wallace, 1972).

The anaerobic responses to activity and hypoxia could be independent in intertidal crabs. The respiratory and biochemical response to hypoxia of *P. crassipes*,

which inhabits high oxygen environments, with that of *Carcinus maenas*, which does encounter environmental hypoxia, can be contrasted. The anaerobic response of *Carcinus maenas* to activity is similar to that of *P. crassipes*; both species fatigue rapidly when running in air and have a similar increase in haemolymph lactate concentrations with activity (Table II).

In *P. crassipes* the rate of aerobic respiration declines with water P_{O_2} . If lactate is the major end product of anaerobiosis, then the aerobic/anaerobic energy balance can be calculated as follows. The energy requirements of a crab submerged for 3 hr in water saturated with air can be estimated:

$$53 \mu\text{l O}_2/\text{g per hr} \times 3 \text{ hr} = 159 \mu\text{l O}_2/\text{g}$$

$$159 \mu\text{l O}_2/\text{g} \times \frac{290 \mu\text{moles ATP}}{1 \text{ O}_2} = 46.1 \mu\text{moles ATP/g}$$

Using the average $\dot{V}_{O_2}$ during the hypoxic period (assuming a linear decrease of $\dot{V}_{O_2}$ with P_{O_2} ; see Fig. 3) and the lactate accumulation during that 3 hr period, the energy production during hypoxia can be estimated:

$$26 \mu\text{l O}_2/\text{g per hr} \times 3 \text{ hr} = 78 \mu\text{l O}_2/\text{g}$$

$$78 \mu\text{l O}_2/\text{g} \times \frac{290 \mu\text{moles ATP}}{1 \text{ O}_2} = 22.6 \mu\text{moles ATP/g}$$

$$0.131 \text{ mg lactate/g} \times \frac{16.7 \mu\text{moles}}{\text{mg lactate}} = 2.2 \mu\text{moles ATP/g}$$

$$\text{total} = 24.8 \mu\text{moles ATP/g}$$

It appears that lactic acid production in *Pachygrapsus crassipes* is insufficient to compensate for the hypoxic condition and may, in fact, be the result of movement within the experimental chamber. The lactate accumulation in the haemolymph of *Carcinus maenas* under similar conditions of hypoxia is relatively larger and may play a more extensive role in maintaining normoxic metabolic rate; in water with low P_{O_2} *Carcinus maenas* completely abandons aerobic respiration and generates energy anaerobically.

It is possible that specialization for lactate production during burst activity canalizes an organism into utilizing that biochemical pathway during environmental hypoxia. Lactate is accumulated by vertebrates during intensive activity and it is also the primary anaerobic end product of vertebrates during hypoxia; a similar pattern appears to exist in the Crustacea. It is presently believed that there is no substrate inhibition of LDH at physiological concentrations of enzyme and substrate (see Vessell, 1972, for review). Thus, given sufficient concentrations of NADH, the presence of LDH will always result in lactate accumulation when oxygen is limited, whether this condition is due to activity or environmental hypoxia.

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SUMMARY

Crabs from different intertidal environments show different anaerobic metabolic responses to hypoxia and activity. I chose to compare *Carcinus maenas*, a species which lives in a variety of habitats and may encounter hypoxic conditions, and *Pachygrapsus crassipes*, which inhabits rocky zones and is not subjected to conditions of reduced oxygen availability. Rates of oxygen consumption ($\dot{V}_{O_2}$) and lactate production were used as indices of aerobic and anaerobic metabolic rate, respectively.

Both species showed similar capacities for anaerobic energy production during activity. Activity was vigorous, of short duration, and accompanied by a rapid rise in haemolymph lactate levels.

Activity in specimens of *P. crassipes* was primarily anaerobic. Burst running speed was constant within an ecologically relevant range of temperature. Aerobic metabolic rate, duration of activity, and maintenance of burst speed were positively correlated with temperature.

Individuals of *P. crassipes* had a decreased metabolic rate during air exposure. Aerial $\dot{V}_{O_2}$ was 35% lower than the rate during submersion, but whole body lactate content was the same. Submerged crabs of both species maintained vigorous activity longer than those exposed to air, suggesting that depressed metabolism can influence activity.

The two species differed in their response to hypoxia. *Carcinus maenas* specimens maintained $\dot{V}_{O_2}$ constant as water P_{O_2} declined from 160 to 50 torr, had a reduced $\dot{V}_{O_2}$ as water P_{O_2} declined from 50 to ca. 20 torr. Below this tension *Carcinus maenas* specimens abandoned aerobic respiration and increased lactate production. *Pachygrapsus crassipes* specimens neither regulated $\dot{V}_{O_2}$ nor increased lactate production under conditions of reduced oxygen availability. Thus, the anaerobic response to hypoxic conditions is independent of the metabolic response to activity in these species, and the metabolic responses of specimens of *Carcinus maenas* appear to be adaptive to the environments which that species encounters.

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FUNCTIONS OF SWIMMING SETAE WITHIN CAPRELLID AMPHIPODS (CRUSTACEA)

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The term "swimming setae" has been given to paired, long, plumose setae arising in a "V" pattern from the ventral surface of the antennae of caprellid amphipods (Figs. 1, 2). Swimming setae are located on the two distal peduncular articles of the antennae and may occur on the proximal flagellar article (Mayer, 1882, 1890, 1903; McCain, 1968; Laubitz, 1970, 1972). Presence or absence of swimming setae on the flagellum is characteristic for a species.

Two functions have been attributed to swimming setae: locomotion (see Wetzel, 1932) and particle capture during filter-feeding (Caine, 1974, 1977). Without quantification Wetzel (1932) considered feeding to be the primary function of swimming setae. No recent analysis has been directed toward locomotory mechanisms of caprellids. However, Keith (1971) indicated directional swimming by two species of *Caprella*. Caine (1977) measured particle capture by caprellids but assumed that presence of swimming setae indicated an equal reliance upon filter-feeding in food acquisition. Subsequent attempts to rear caprellids with swimming setae showed that some species survived only when periphyton was present. Survival of some other species was independent of periphyton presence.

Only four genera of caprellids have swimming setae (*Caprella*, *Heterocaprella*, *Metacaprella*, and *Tritella*), but 127 of the 241 described species of the suborder Caprellidea (excluding the whale lice, Cyamidae) belong to these genera. *Caprella* alone has 119 described species, and it is the only genus in the Caprellidea with more than 9 described species.

It is possible that swimming setae had some impact on the species proliferation of *Caprella*. This study was undertaken to quantify the importance of swimming setae to swimming ability and to critically examine filter morphology.

MATERIALS AND METHODS

Swimming rates were measured in the laboratory. Specimens in a running seawater aquaria were released 10 cm from an *Obelia dichotoma* (L.) colony (a naturally occurring substratum) and time from the onset of swimming until the substratum was reached was recorded. Species with swimming setae were *Caprella laciniuscula* Mayer, *Caprella natalensis* Mayer, *Caprella striata* Mayer, *Metacaprella kennealyi* (Stimpson), and *Tritella pilimana* Mayer; species without swimming setae were *Deutella californica* Mayer and *Paracaprella tenuis* Mayer. *P. tenuis* is an Atlantic species and was tested with *Bugula avicularia* (L.) as a substratum. Specimens were released horizontal to the substratum sample and

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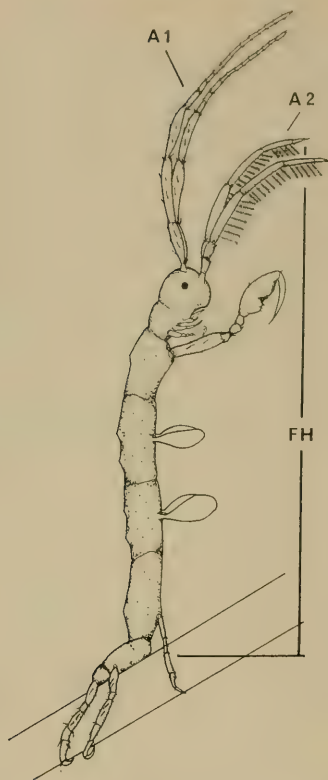


FIGURE 1. Upright stance of a generalized caprellid. Swimming setae are present on the two distal peduncular articles and on the proximal flagellar article of the antennae. A1 represents antennules, A2 represents antennae, and FH represents filtering height.

lighting was indirect and "uniform" shaded sunlight, reducing phototactic responses. Due to the presence of the brood pouch, sexes were analyzed separately; 50 specimens of each sex were tested for each species.

The five species with swimming setae were reared in the laboratory. Oviparous females of a species were placed on various substrata and their broods were allowed to develop, emerge from the brood pouch, and grow for six weeks. The biotic substrata were red algae (*Odonthalia floccosa*, *Rhodomela laria*, and an

unidentified filamentous species), brown algae (*Fucus disticus* and *Sargassum multicum*), and hydroids (*Abietinaria* sp., *Aglaophenia* sp., and *Obelia dichotoma*). Algal substrata supported little periphyton and entrapped minimal detritus, while hydroids supported abundant periphyton and the periphyton aided in entrapping detritus. Relative abundances of periphyton and detritus were subjectively determined.

Survivorship on different substrata was most different between *Caprella laeviuscula* and *C. natalensis*. Morphological variations of the antennae were examined in detail for these two species. Ultrastructure was observed using standard histo-

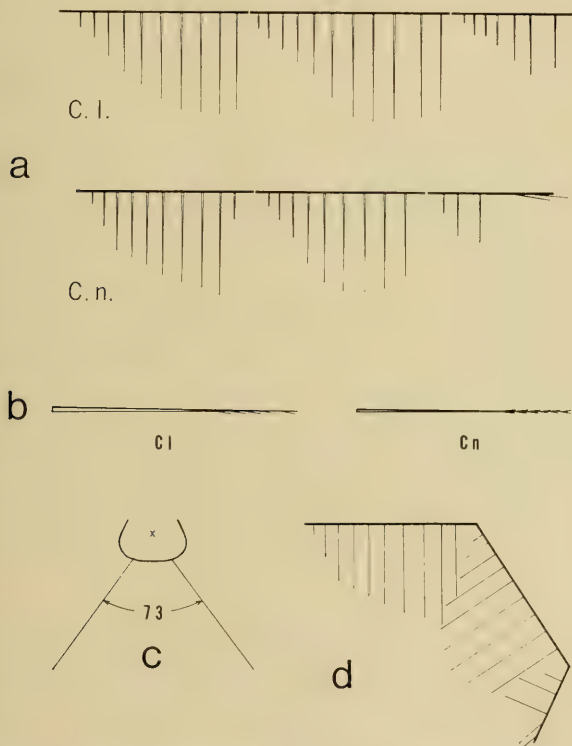


FIGURE 2. Diagrammatic representation of spacing of swimming setae on the distal peduncular and proximal flagellar articles of the antennae of *C. laeviuscula* (C. l.) and *C. natalensis* (C. n.) (a); the individual swimming setae (b); the antennal cross section indicating setal angle (c); and the crescent-shaped filter formed by the antennae (d).

TABLE I

Comparative swimming rates of caprellids with swimming setae before and after swimming setae removal. Rates in cm s^{-1} .

	With swimming setae		Swimming setae removed	
	Males	Females	Males	Females
<i>Caprella laeviuscula</i>	0.70 ± 0.14	0.59 ± 0.10	0.64 ± 0.19	0.54 ± 0.10
<i>Metacaprella kennealyi</i>	2.00 ± 0.10	2.38 ± 0.17	1.79 ± 0.14	2.17 ± 0.24
<i>Tritella pilimana</i>	0.90 ± 0.15	1.02 ± 0.14	0.83 ± 0.18	0.92 ± 0.17

logical techniques with the antennae fixed in Gilson's solution, imbedded in paraffin, serially sectioned at $10 \mu\text{m}$, and stained in Mallory's triple stain. Sections were examined for indications of structures or innervation that would allow setal movement of affect filter selectivity.

Measurements of spacing between the setal bases and setal length were made of 75 specimens of each species with the aid of an ocular micrometer. Measurements were also made of filtering height (antennal length plus body length minus pereonites VI and VII), body length, and length of each antennal article (Fig. 1). Filtering area of the antennae was computed as the sum of the areas bounded by adjacent setae, where each area is the product of the shorter seta length and the distance between setae.

For each specimen, regression plots of the fraction of total filtering area at a particular size was made. The plot indicated the portion of the filter that captured particles of a specified size, or particles larger than that size: the per cent filtering area vs setal spacing (% FA:SS). Regression data for each specimen were then combined for the species.

Natural particle size distribution was measured with a Model B Coulter-Counter by oceanography classes at Friday Harbor Laboratories during the summers of 1970, 1972, and 1974. Although Coulter-Counters read the volume of chain-forming cells as the volume of the chain, filters engage the chain and not each cell. Hence, the inaccuracy of measurements is only important in computing actual size: frequency distributions of particles (the volume of the cells will be overestimated by a Coulter-Counter). Corrections for volume of chain-forming cells were not computed for this study.

RESULTS

Only *Caprella laeviuscula*, *Metacaprella kennealyi*, and *Tritella pilimana* consistently swam toward the substratum sample upon release. The other species sank either in an outstretched position or in a compact shape, bending ventrally with the anterior and posterior pereonites touching. Removal of swimming setae from those species that swam resulted in a $9.0 \pm 1.0\%$ reduction in swimming rate (Table I).

Setal insertions are rigid and no muscles were found that inserted on the setae. Gross antennal musculature agree with findings of Wetzel (1932). Length of swimming setae progressively increase distally to a maximum length and then

decrease. This pattern of setal length reduces overlap among the swimming setae when the antennae are flexed (Figs. 2a, d). Between the peduncular articles with swimming setae (antennal articles three and four), an angle of $54.5 \pm 1.7^\circ$ must be exceeded for the setae to intermesh; setal overlap occurs if the articulation between the peduncle and flagellum exceeds $57.0 \pm 0.8^\circ$. By flexing the antennae slightly less than the critical setal overlapping angles, a crescent-shaped filtering structure is formed (Fig. 2d). Swimming setae form an angle of 73° (Fig. 2c). The distal $31.1 \pm 1.4\%$ of each swimming seta is plumose, but the setule placement varies interspecifically. Setules on the swimming setae of *C. laeviscula* are on the medial side only while setules of *C. natalensis* are on the lateral sides of swimming setae (Fig. 2b). Maximum space between a setule and the seta is $2 \mu\text{m}$ for *C. laeviscula* and $1 \mu\text{m}$ for *C. natalensis*.

Analysis of the % FA:SS data for *C. laeviscula* yields a linear relationship for a given body size ($\bar{r}^2 = 0.933 \pm 0.050$). However, as size increases, the slope of the % FA:SS regression decreases logarithmically and the X-intercept increases logarithmically (Table II). The % FA:SS regression for *C. natalensis* is logarithmic for body size ($\bar{r}^2 = 0.960 \pm 0.43$) and, as body size increases, the slope decreases logarithmically and the X-intercept increases logarithmically. The overall regression for *C. natalensis* for the determination of the slope of the % FA:SS line as body size increases has a slope of -0.380 , thereby indicating a narrower range of setal spacing (regression for *C. laeviscula* has a slope of -1.007 , Table II). Particle encounter of the two setal patterns is indicated in Figure 3.

The antennal figures only depict a single row of setae. As there are a pair of antennae, there are four rows of similar setae. Filtering area (Table II) is then $4(SS_i \times SL_i)$ where SS_i equals setal space_i and SL_i is the length of the shorter of the two space_i-defining setae.

Maintenance experiments indicate that all sizes of *C. natalensis* are capable of surviving on the test substrata. Large *C. laeviscula* survived on all of the test substrata, but specimens 5 mm or less were incapable of surviving on the test algal substrata (none supported dense growths of diatoms). If colonies of diatoms, e.g., *Navicula* sp. and others, were added to the maintenance tests, *C. laeviscula* of all sizes survived on all substrata.

DISCUSSION

Swimming abilities vary among caprellid species and presence of swimming setae is not, in itself, indicative of frequent swimming, but swimming setae do aid in swimming rates of the species examined herein (Table I). A similar swimming rate decline may occur in removal of setae from the antennae of swimming caprellids lacking swimming setae. Many species of caprellids occur in the plankton (McCain, 1968), especially in the water column over natural habitats such as grassbeds (Hobson and Chess, 1976; Williams and Bynum, 1972). These plankton samples include species with and without swimming setae. The implied relationship between swimming setae and swimming ability, therefore, is questionable.

Setation of the antennae and mouthparts of caprellids is related; if swimming setae are present, then the maxillipeds and maxillae are setose. It was previously

TABLE II
Lists of formulae describing size relationships and filtering analysis of C. laeviuscula and C. natalensis.

	<i>Caprella laeviuscula</i>		<i>Caprella natalensis</i>	
	Regression	r ²	Regression	r ²
¹ Body length:Filtering height	1.16 X ^{0.972}	0.999	1.05 X ^{1.007}	0.991
¹ Antennae article 3:Body length	0.091 X ^{0.888}	0.970	0.102 X ^{0.779}	0.958
¹ Antennae article 4:Body length	0.108 X ^{0.805}	0.969	0.098 X ^{0.784}	0.978
¹ Antennae article 5:Body length	0.082 X ^{0.713}	0.971	0.056 X ^{0.907}	0.968
² Smallest setal spacing:Filtering height	15.10 X ^{0.466}	0.932	11.17 X ^{0.396}	0.939
² Filtering Area*:Filtering height	(3-21 mm) 0.528 X ^{2.20}	0.995	(3-14.3 mm) 1.508 X ^{1.112}	0.990
² Individual Regression for:				
Slope				
X—intercept	Linear 20.99 X ^{-1.007}	0.900	Log 11.43 X ^{-0.380}	0.936
Overall Regression Correlation	Linear 15.10 X ^{0.466}	0.932	Log 11.17 X ^{0.396}	0.939
Percentage of Overall Filtering Area:		0.933 ± 0.050		0.960 ± 0.043
Antennae Article 3		39.8 ± 2.2		42.1 ± 2.6
Antennae Article 4		49.5 ± 2.9		49.1 ± 3.1
Antennae Article 5		10.7 ± 1.3		8.8 ± 4.2

* × 10⁴ μm².

¹ X = body length.

² X = filtering height.

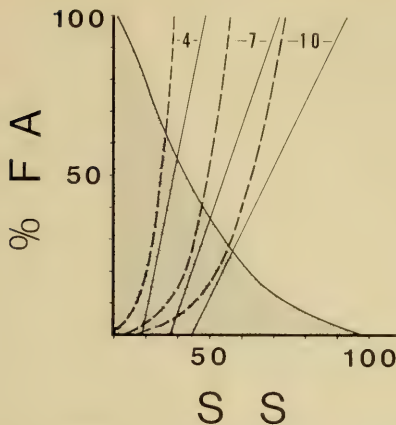


FIGURE 3. Per cent filtering area. Setal spacing in μm for 4, 7, and 10 mm filtering height specimens of *C. lacviuscula* (solid lines) and *C. natalensis* (broken lines) as determined by equations in Table II. Nearshore particle distribution (shaded) indicates the percentage of particles between 20 and 97 μm that is larger than the SS value.

postulated that the presence of swimming setae indicated that filter-feeding was the primary means of acquiring food (Caine, 1974, 1976, 1977). However, it now appears that this was an overgeneralization.

The relative importance of periphyton to the two species investigated is indicated in their survival on algal substrata. *C. lacviuscula* survived on those substrata supporting dense epiphyte growths but did not survive on those substrata lacking epiphytes (red and brown algae). Several factors could explain the failure of *C. lacviuscula* to maintain itself on these substrata in the field, including algal release of specific toxic substances, algal microhabitats occurring outside the caprellid's tolerance limits, selective predation, emigration, or lack of food. In controlled laboratory experiments, only the release of specific toxic substances and lack of food remain possible explanations. Due to the species survival with the addition of colonial diatoms, it is contended here that death is due to the lack of periphyton on the substrata. Similarly, the survival of *C. natalensis* on these algal substrata indicates the relative unimportance of periphyton to this species and reliance on suspended particles and filter-feeding. The mechanism(s) whereby periphyton is retarded or excluded from the algae is beyond the scope of this project, but Sieburth and Conover (1965) have found that tannins released from brown algae retard the survival of algal, including diatom, and hydrozoan epibionts.

By comparing the nearshore particle distribution with filter space:filter area relationships, it becomes apparent that *C. natalensis* has the ability to filter more abundant material than does *C. lacviuscula*. Although the differences appear slight, they are significant. For example, a model specimen of *C. natalensis*, 4 mm filtering

height, retains 100% of the particles between 38.2 and 97 μm entering its filter, whereas only 49% of the particles between 38.2 and 97 μm are retained by a model specimen of *C. laciniuscula* (Fig. 3). *C. natalensis* retains 58% of the particles between 20 and 97 μm , while *C. laciniuscula* retains only 37% of the similarly sized particles, i.e., *C. laciniuscula* is only 63% as efficient as *C. natalensis* in filtering particles in the size range at 4 mm filtering height. Setal spacing differences appear to be less at smaller setal spacing, but 50% of the filtering area of *C. laciniuscula* is between 29 and 38 μm (19% of the particles between 20 and 97 μm) while 50% of the filtering area of *C. natalensis* is between 20 and 34 μm (33% of the particles between 20 and 97 μm). Thus, retention differences are still about 58% when considering just the lower 50% retention levels.

At 10 mm filtering height, *C. natalensis* captures 100% of the particles 73.1 μm (10% of the particles between 20 and 97 μm) and *C. laciniuscula* filters 100% of the particles 92.5 μm (3% of the particles between 20 and 97 μm). Filtering differences continue as particle occurrence continues to decrease logarithmically as particle size increases (Sheldon, Prakash, and Sutcliffe, 1972).

During Stage D of the molt cycle, if the amphipod molt cycle is similar to that of decapods, the procuticle of the new exoskeleton is formed beneath the old exoskeleton. The new procuticle follows the outline of the old exoskeleton, including setae. Therefore, the space between the setae expands as the caprellid expands upon molting. One possible explanation for the antennal morphologies (linear vs. logarithmic) is that each seta is added proximally on the antennal articles of *C. laciniuscula*. Every setal space would increase with each molt, resulting in a linear pattern. A logarithmic spacing could be maintained if a seta could be added either proximally or distally on the antennal article; distal additions would tend to compress the setal spaces. An alternate explanation is that growth of the antennae influences the setal spacing (Table II). The antenna of *C. laciniuscula* grows faster than that of *C. natalensis*. A third possibility is a combination of both suggestions; differential growth rates and variations in setal additions.

Field data support the periphyton requirement of *C. laciniuscula*. The lower depth of limit of *C. laciniuscula* in Puget Sound approximates the lower depth limit of colonial diatoms (ca. 25 m) (R. Shimek, personal communication; Caine, unpublished) and populations of *C. laciniuscula* are most dense in those areas supporting dense growths of colonial diatoms (quiet waters).

Rubenstein and Koehl (1977) have approached biological filters as an engineering and mathematical problem. They suggest that filters may capture particles not only by sieving, but also by direct interception (particle adherence upon contact, as with mucus), inertial impaction, gravitational deposition, motile particle deposition, and electrostatic attraction. Only sieving and direct interception are used by caprellids in filter-feeding (predation includes, but is not limited to, motile particle deposition). However, the type of water currents filtered by a caprellid appears to be a function of the habitats occupied. If wave- and tide-generated currents are strong enough (between 2 and 15 cm s^{-1}), caprellids usually filter the water currents passively. This is accomplished by maintaining an upright stance with the ventral surface oriented into the current. Lower currents require self-generated currents, either by a rocking motion of the entire animal which pulls the antennae through the water (Caine, 1974), or by beating of the maxillipeds

so that an antero-dorsal current through the antennal filters is produced (Caine, 1977). In currents greater than 15 cm s^{-1} , caprellids grasp the substratum with the pereopods and gnathopods, thereby holding their bodies against the substratum.

Thus, caprellid control over particle capture by varying the velocity of self-generated currents only occurs at currents less than 2 cm s^{-1} . It is contended here that particle retention is only marginally affected by such slow currents. Within the range of filterable currents, flow patterns are not uniform through the biotic substrata and a caprellid may choose those parts of the microhabitat that yield maximum rate of capture of particles. If there is competition for those areas of the microhabitat, then intra- and interspecific competition should be manifest.

The efficiency of particle capture by direct interception can also be modified by using an adherant substance on the filter. Caine (1974) attributed such an adherant substance to the caprellid, but no secretory organs were found in the antennae, and mucus only occurred when the caprellid frequently brushed the antennae against the gorgonian (Cnidaria) host. The presence of mucus on the antennae results in the retention of materials finer than the setal spacing, thereby improving the efficiency of filtering. However, this has only been observed with *Caprella penantis* Leach on *Leptogorgia* sp.

Between feeding and locomotion, swimming setae are more important in the acquisition of food. The concomitant increase in mouthpart setation with swimming setae allow the caprellid species to filter particulate matter from the water, scrape and retain material from the substratum, or both filter-feed and scrape. The scraping/filter-feeding lifestyle deviates from the predatory lifestyle of caprellids without swimming setae (Caine, 1977), and the presence of swimming setae is considered to be an advanced condition (McCain, 1970).

Perhaps this feeding ability is related to the species explosion of *Caprella*. If such is the case, why did *Caprella* diversify while the other genera did not? *Tritella* retains a mandibular palp and, rather than use the setose mouthparts to glean entrapped material from the antennae, the mandibular palps clean the swimming setae (Caine, 1976). With relatively inefficient antennal cleansing, *Caprella* should be a better competitor for food. The case with *Metacaprella* is not nearly so straightforward. *Metacaprella* is a large caprellid that is usually found in relatively shallow water (Laubitz, 1970). *M. kennerlyi* is aggressively subordinate to *C. laeviuscula* (Caine, unpublished) and the combination of selective predation by fish and competition for food and space with *Caprella* may limit *Metacaprella* speciation.

SUMMARY

1. All caprellids with swimming setae do not swim, but of those that do, a 9.0% decrease in swimming rate was measured after swimming setae removal. The literature indicates that many caprellids without swimming setae are found in the plankton, and presumably swimming. The importance of swimming setae to the ability to swim is not absolute.

2. Rearing attempts of caprellids with swimming setae showed that some species required periphyton for survival while other species could sustain themselves by filter-feeding. If periphyton was added to the trials, all species survived.

3. Regression analyses indicated two patterns of swimming setal spacing. Linear regressions gave the best fit of cumulative filtering area: setal spacing lines for those species requiring periphyton. Those species surviving only with filterable material were best fit with log regressions.

4. Particle frequency in Puget Sound is constantly decreasing as particle size increases. The log pattern of setal spacing more than doubles the amount of material filtered over the linear pattern.

5. *Caprella* has 49% of the described species of caprellids; the next most numerous genus has 4%. Swimming setae opened a new feeding niche for caprellids and this led to the species explosion. Other genera with swimming setae are competitively subordinate to *Caprella*.

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DEVELOPMENT OF THE DIMORPHIC CLAW CLOSER MUSCLES OF THE LOBSTER *HOMARUS AMERICANUS*. IV. CHANGES IN FUNCTIONAL MORPHOLOGY DURING GROWTH

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A distinctive feature of many crustaceans is their dimorphic chelipeds. These asymmetric structures appear to have co-evolved many times with a variety of behavioral adaptations. For example, an adaptation for sexual behavior is reflected by the presence of the striking sexual dimorphism of the chelipeds in fiddler crabs (Crane, 1975) and the less obvious one in crayfish (Stein, 1976). Other species exhibit adaptations for defense or prey-capture behaviors and thus have dimorphic chelipeds common to both sexes (Shoup, 1968; Ritzmann, 1974; Hamilton, Nishimoto, and Halusky, 1976; Lang, Govind, Costello, and Greene, 1977). The occurrence of dimorphism poses interesting questions in relation to the developmental changes that occur during formation of these specialized limbs. In this regard, claw asymmetry falls into two categories: fixed and plastic (Przibram, 1931). If the species is in the latter category, then each claw maintains the capability of expressing either limb type on either side throughout its life cycle. If the larger claw is lost, the remaining smaller claw undergoes a change to become the larger one, a process called "reversal". The new claw regenerates as the smaller claw (Przibram, 1931; Shoup, 1968; Lewis, 1969; Hamilton, Nishimoto, and Halusky, 1976; see also Mellon and Stephens, 1978).

On the other hand, for species in which claw type is fixed, loss of either claw will result in regeneration of the same type claw (Przibram, 1931; Huxley, 1932). Such is the case in the adult lobster *Homarus americanus* (Herrick, 1896; Emmel, 1908). The adult lobster possesses a narrow cutter claw (pincer) and a broad crusher. Removal of one results in regeneration of the same type claw. However, the claw type is fixed only after the fifth stage (approximately 20-30 days after hatching). Prior to this stage, the claws are symmetrical in appearance and each is capable of developing into either claw (Emmel, 1908; Lang, Govind, and Costello, 1978).

In addition to the changes which occur in external morphology during growth, there are also changes in the amount of fast and slow muscle in the claws. In the adult lobster, the two closer muscles differ from one another (Jahromi and Atwood, 1971; Goudey and Lang, 1974). The phasic-type cutter claw has a

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closer muscle composed of 65% fast fibers (2–4 μ m sarcomere lengths) while the remainder are slow fibers (6–12 μ m sarcomere lengths). The tonic-type crusher claw contains only slow fibers (over 6 μ m sarcomere lengths).

In the larval stages (1–3) and early postlarval stages (4–5), the claw's external symmetry parallels an internal symmetry of fiber types in the closer muscles. Until late stage 5, both claws possess closer muscles containing about 30% short sarcomere fibers. The remainder of the muscle consists of intermediate to long sarcomere fibers. After the 5th stage the number of short sarcomere fibers rapidly increases in one claw and slowly decreases in the other claw. The trend continues until about the 16th stage, when the closer muscles have attained their adult distribution of fibers (Lang, Govind, and She, 1977; Govind and Lang, 1978).

The changes in morphology of the claws and of their closer muscles from the postlarval symmetrical condition to the adult dimorphic state probably reflect their changing roles in defense and prey capture behaviors (Lang, Govind, Costello and Green, 1977). For instance, Warner and Jones (1976) and Vermeij (1977) demonstrated that the differing structures of the dimorphic claws in several crab species provide varying degrees of mechanical advantage. In addition, cheliped structure was related both to the type of muscle fibers in the closer muscle and to the crab's prey.

The present paper describes changes in several morphological characteristics of the claws during the growth of the lobster. It was found that the closer muscle is arranged into discrete bundles of fibers. In the adults each bundle consists of a homogenous population of fast or slow muscle fibers. In addition, the mechanical advantage of the claws is shown to be related to the fiber content of the groups found in the closer muscles. All these characteristics are congruent between the paired claws of early postlarval lobsters (stages 4–5) but they commence to diverge in late stage 5 and stage 6 as part of the maturation process which produces the specialized, asymmetric chelipeds of the adult.

MATERIALS AND METHODS

Larval lobsters (*Homarus americanus* Milne-Edwards) were obtained from the Massachusetts State Hatchery, Martha's Vineyard, and reared in circulating sea water tanks at ambient temperatures (20–23° C) (Hughes, Shleser, and Tchohanoglous, 1974). When the animals molted into the first juvenile stage (stage 4), they were placed in individual trays (Lang, 1975) to monitor subsequent growth. Adult lobsters were obtained locally from the waters around Woods Hole, Massachusetts. The animals were kept in tanks supplied with fresh running ambient sea water. Juvenile animals were fed 2–3 times daily, adult animals 3 times each week.

No claw restraints were used on lobsters of any age. The animals were weighed prior to claw removal without completely drying them. Length measurements were made with a set of calipers. For small lobsters, a dissecting microscope with a calibrated ocular micrometer was used. Total length was taken as the distance from the tip of the rostrum to the end of the telson.

The closer muscles were fixed with alcoholic Bouin's in a manner similar to that described previously (Lang, Costello, and Govind, 1977). The dactyl of



FIGURE 1. Closer muscles from the cutter claw (top, medial view) and the crusher claw (lateral view) of a lobster (3100 g). Muscle is oriented such that the proximal side is to the right and the dorsal side is to the top. Dactyl insertion is marked by an asterisk. Scale: 4 cm.

the claw was held in the fully open position during fixation. The muscle was perfused for 2-4 hours with fresh fixative by inserting hypodermic needles into holes drilled through the exoskeleton. In the case of the smaller lobsters, slits were made in the exoskeleton dorsal to the opener muscle and ventral to the closer muscle to permit good infiltration, and fresh fixative was continually added during the fixation period. The opener muscle was then removed and the claw with the closer muscle, *in situ*, was kept in fresh alcoholic Bouin's for 18-24 hours. The closer muscle was isolated by removing the exoskeleton and any connective tissue or hemolymph covering it. It was then placed in 90% alcohol.

Fully exposing the muscle showed that it was separated into physically distinct fiber groups at all stages (Fig. 1). Subsequently, all sampling of muscle fibers was based on these groups. A minimum of ten muscle fibers was taken from each exposed group on the medial side of the claw. In the case of the larger groups, ten fibers were taken from a proximal section and from a distal section. In one animal from each postlarval stage, sampling included an additional ten fibers from the central portion of the larger groups.

TABLE I

Distribution of muscle fiber types in the paired claw closer muscles of the lobster.

Stage (No.)	Length (cm)	Weight (g)	Muscle fiber types based on sarcomere length (μm)					
			Claw I/Cutter			Claw II/Crusher		
			2-4 (Fast)	4-6 (Intermediate)	>6 (Slow)	2-4 (Fast)	4-6 (Intermediate)	>6 (Slow)
4 (3)	1.3	<0.2	29%	15%	56%	29%	23%	48%
5 (2)	1.4	<0.2	43	10	47	33	3	64
5 late (1)*	1.4	<0.2	64	7	29	32	10	58
6 (3)*	1.6	<0.2	59	9	32	24	3	73
Adult (1)*	17.8	209	56	0	44	0	5	95
Adult (1)*	20.3	271	56	1	43	—	—	—
Adult (1)*	20.3	310	57	0	43	—	—	—
Adult (1)*	41.7	3100	28	31	40	0	0	100

Minimum of 70 fibers sampled from each claw.

* Significant difference between paired claws at 0.01 level (Kolmogorov-Smirnoff two-sample test).

The sarcomere lengths were measured in the same manner described in Lang, Costello, and Govind (1977). As in earlier papers, classification of fibers was based on sarcomere length (Jahromi and Atwood, 1971; Goudey and Lang, 1974). Fibers with a sarcomere length less than $4 \mu\text{m}$ are considered fast, those with sarcomere lengths from $4-6 \mu\text{m}$ are intermediate, and those whose sarcomere length is greater than $6 \mu\text{m}$ are slow. The correlation between sarcomere length and contraction speed has been demonstrated in adult lobsters (Jahromi and Atwood, 1971) but not in juvenile animals. However, other factors being equal, a fiber with more sarcomeres per unit length will contract more rapidly than one with fewer sarcomeres (Huxley and Niedergerke, 1954; Josephson, 1975). Differences in the populations of muscle fiber types between paired claws were tested using the Kolmogorov-Smirnoff two-sample test (Siegel, 1956).

The distribution of fast and slow muscle fibers in the adult cutter claw was determined from dry weight measurements of each major muscle group. Nine closer muscles were fixed in alcoholic Bouin's in the manner previously described. All the fibers of each group on the lateral and medial sides of the claw were weighed. The dry weight of each group was used to derive its percentage contribution to the muscle mass.

The mechanical advantage of the claws was calculated by utilizing the techniques described by Alexander (1968). The efficiency in transferring the force from contraction of the closer muscle to the closure of the dactyl depends, in large part, on the mechanical advantage of the dactyl. Assuming a frictionless system, $F_1L_1 = F_2L_2$ where F_1 is the force applied, F_2 is the force produced and L_1 and L_2 are the lengths of the lever arms (see Fig. 2). L_1 is the distance from the pivot point of the dactyl to the point of insertion of the apodeme; L_2 is the distance from the pivot point to the tip of the dactyl. The mechanical advantage can be calculated as a ratio of L_1 to L_2 . Measurements were made to the nearest 0.1 mm.

RESULTS

The closer muscles of both adult and juvenile claws were examined to determine the exact locale of fiber types and the presence of regional grouping of the fibers. Thus it was possible to determine the differential changes of fiber types which occurred within each part of the closer muscle at successive molts.

To gain a clear picture of the overall and of the localized changes of fiber types in the maturing closer muscle, two different methods were used to catalog muscle type distribution. First, the average distribution of fiber types was determined for the entire closer muscle from the paired claws of the animals (Table I). Second, the localized transformations of fiber type were determined for each closer muscle group (Table II). However, claw type is not recognizable in the early postlarval stages (4-5); therefore the closer muscle with the higher percentage of fast muscle is labeled Claw I, and the other Claw II. At stage 6 and thereafter, the claw type can be distinguished and is so labeled. The results are presented first for the adults and then separately for each of the early postlarval stages.

Adult

The composition of the closer muscles is shown in Table I. Although a different sampling method was employed, the relative distribution of the fiber types did not differ markedly from that reported earlier (Jahromi and Atwood, 1971; Lang, Costello, and Govind, 1977). The cutter closer muscle contains both fast and slow fibers. The crusher has only slow fibers. Using the identical sampling method for all sizes of lobsters (200-3100 g), the percentage composition of the fast and slow fibers in the cutter closer muscle remained essentially similar in the lobsters examined. Thus, the adult pattern, once established, apparently remains constant even though the muscle itself enlarges greatly, probably by increasing fiber diameter, the number of fibers and the number of sarcomeres within a fiber (Govind, She, and Lang, 1977).

Cutter claw closer muscle morphology. When the entire closer muscle was fixed and then exposed by removal of the exoskeleton, it became apparent that it was divisible into several distinct groups or bundles of fibers (Fig. 1). These groups were easily separable based on two criteria: first, there were spaces between the bundles, which formed distinct boundaries, and second, the angle of insertion by the fibers onto the apodeme differed among bundles of fibers. Fibers within the same group had similar insertion angles.

The groups were labeled according to their position and size (Fig. 3). The largest of the groups, CuD, was divisible into dorsal and ventral subunits, 1 and 2, respectively. This division was more prominent in some animals than in others. Likewise, the proximal group, CuP, was divided into subunits 1 and 2. The relative sizes (dry weight) of CuP1 and CuP2 varied from one animal to another. In most cases CuP1 was almost twice as large, but in one animal it was 4.9 times that of the other. CuPV was always the smallest of the groups in the closer muscle.

The groups CuD and CuV were bilaterally symmetrical with respect to the apodeme. The fibers in the former group inserted onto the apodeme at an angle

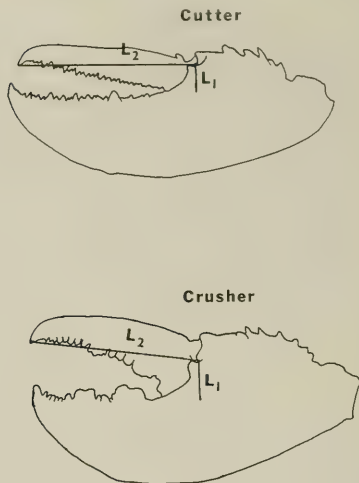


FIGURE 2. Propodites of lobster claws showing lengths used to calculate the mechanical advantage at the dactyl tip (L_1/L_2). L_1 is the distance from the pivot to the insertion of the apodeme onto the dactyl; L_2 is the distance from the pivot to the dactyl tip.

of about 28° while those in the latter group inserted at a much more acute angle (generally 12°). The fibers in group CuP inserted onto the apodeme at its most proximal edge with no separation into lateral or medial fibers. CuPV inserted only on the medial side of the proximal ventral edge of the apodeme. In this respect, the fibers of these proximal groups inserted like a parallel-fibered muscle to form longitudinal units instead of the typical pinnate insertion of CuD and CuV. The angle of insertion by fibers in CuP ranged from a maximum of 6° (outermost fibers) to 0° . In CuPV, the range was from 8° to 0° . These proximal groups probably serve as supplementary muscle by occupying the space characteristic of the posterior aspect of pinnate muscle.

Distribution of fiber types in the cutter claw. The average sarcomere length of fast fibers in the cutter claws of the 209, 271, and 310 g lobsters was $2.6 \mu\text{m}$; for slow fibers, the average was $8.8 \mu\text{m}$. For the 3100 g lobster, the mean sarcomere lengths of both fiber classes were somewhat greater: $3.9 \mu\text{m}$ for fast fibers and $11.2 \mu\text{m}$ for slow fibers. One point to be noted is the apparent paucity of intermediate fibers in the mid-size range lobsters. Sarcomere lengths in these muscles fell generally into two categories: less than $4 \mu\text{m}$ or more than $6 \mu\text{m}$. In the largest lobster, though each mode had a larger average, a major gap still remained between the two types of fiber populations (no sarcomeres were found whose lengths fell between $5.5 \mu\text{m}$ and $10.0 \mu\text{m}$; there was no break in sarcomere lengths ranging from 3.0 to $5.5 \mu\text{m}$).

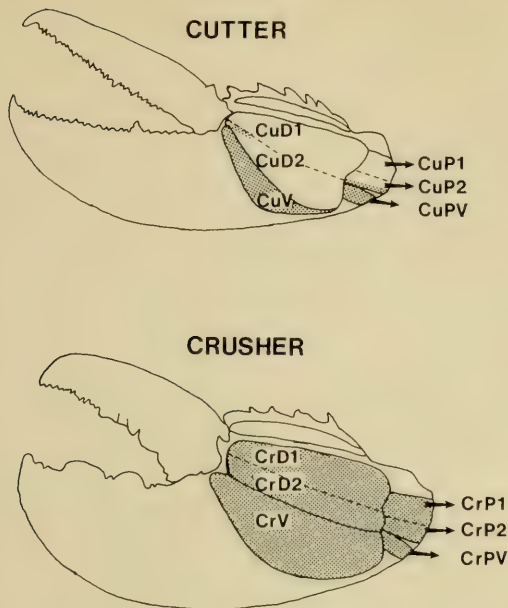


FIGURE 3. Medial aspect of cutter and crusher claws showing the division of the closer muscle into groups of fibers. Stippling denotes slow muscle. Cu indicates Cutter; Cr indicates Crusher; D indicates dorsal; V, ventral and P, proximal.

The probability that a cutter claw closer muscle fiber would be fast or slow was dependent upon the bundle from which it was removed (Fig. 3). Thus, for every cutter claw examined ($N = 4$) each group was found to be essentially homogeneous with regard to its fiber type. CuD and CuP were composed of fast muscle; CuV and CuPV were composed of slow muscle. In the distal region of CuD, occasionally there were isolated slow fibers. These were restricted to a small area in this region of the muscle. On the ventral surface of CuP2 sometimes there were fibers with sarcomere lengths $> 5 \mu\text{m}$; these were few in number and only immediately adjacent to CuPV. Elsewhere in CuP the fibers always contained sarcomeres with lengths $< 4 \mu\text{m}$.

From dry weight measurements of the groups, the closer muscle mass was found to be comprised of 81% short-sarcomere fibers and 19% long sarcomere fibers. The relative composition for the major groups in the entire muscle was 27% (CuD medial): 8% (CuV medial): 32% (CuD lateral): 12% (CuP): 3% (CuPV). The fast fiber content was somewhat higher than previously reported (Goudey and Lang, 1974; Lang, Costello and Govind, 1977). This was probably

due primarily to two factors: first, variability of sampling regions from specimen to specimen in these previous studies, and second, the weight measurements do not reflect fiber numbers (fast fibers tend to have larger diameters and longer lengths).

Crusher claw closer muscle morphology. Although the crusher claw contains only slow type fibers, it too is partitioned into distinct bundles (Figs. 1 and 3). However, the larger distal bundles differ from those in the cutter. The prominent boundary runs longitudinally to divide this large portion into roughly equal halves, CrD and CrV. Occasionally a faint division of the dorsal bundle into subunits 1 and 2 (dotted line in Fig. 3) could be seen. The insertion angles made by the fibers of CrD and CrV onto the apodeme, so markedly different in the cutter, were nearly identical in the crusher (avg. 30° and 27° , respectively). The proximal bundles were similar in size and shape to those of the cutter. The insertion angles made by the fibers of these groups onto the proximal edge of the apodeme were small. Although the maximum angles for the outermost fibers of CrP and CrPV were, respectively, 7° and 9° , the angle for most fibers ranged from much less than this value to 0° .

Distribution of fiber types in the crusher claw. It has been previously shown that all fibers of the crusher claw are of the slow type (Jahromi and Atwood, 1971; Goudey and Lang, 1974; Lang, Costello and Govind, 1977). For this reason the fibers did not differ markedly in sarcomere length among the bundles (Fig. 3). As in the cutter claw, the lengths of the sarcomeres were somewhat greater in the 3100 g lobster. The mean sarcomere length in the 209 g lobster was $7.5 \mu\text{m}$, while that in the 3100 g lobster was $11.0 \mu\text{m}$. In the 209 g lobster, some fibers with intermediate sarcomere lengths ($4\text{--}6 \mu\text{m}$) were observed but there was no apparent bimodal distribution of intermediate and slow fibers. In the 3100 g lobster, no fibers in the crusher claw had sarcomere lengths less than $9 \mu\text{m}$.

Juveniles

The terminology to describe the closer muscle groups is the same as that used for the adults. However, since claw type cannot be discerned prior to stage 6, C— is used in lieu of Cu or Cr (Fig. 4).

Stage 4. In this, the first postlarval stage, the lobster resembles a diminutive adult, although both claws remain identical (Herrick, 1896, 1911). The closer muscle of these claws was divided into groups of fibers. The size and shape of the groups, so dissimilar between closer muscles in the paired adult claws, were identical at this stage (Fig. 4). The dorsal groups, C—D and C—P, contained fast, intermediate and slow fibers (Fig. 5). The ventral groups, C—V and C—PV, contained only long sarcomere fibers (avg = 79%) and intermediate sarcomere fibers in both claws. The symmetry of the closer muscles is clearly reflected by comparing the percentages of short sarcomere fibers in the paired claws (Table I). The average sarcomere length of the short sarcomere fibers in these claws was $2.5 \mu\text{m}$; that of slow fibers was $6.9 \mu\text{m}$.

From Table I, the overall percentage of fast fibers in the whole muscle averaged 29% for both claws. However, this figure included the ventral groups (C—V and C—PV) which apparently have no fast fibers. When the fast fiber and intermediate/

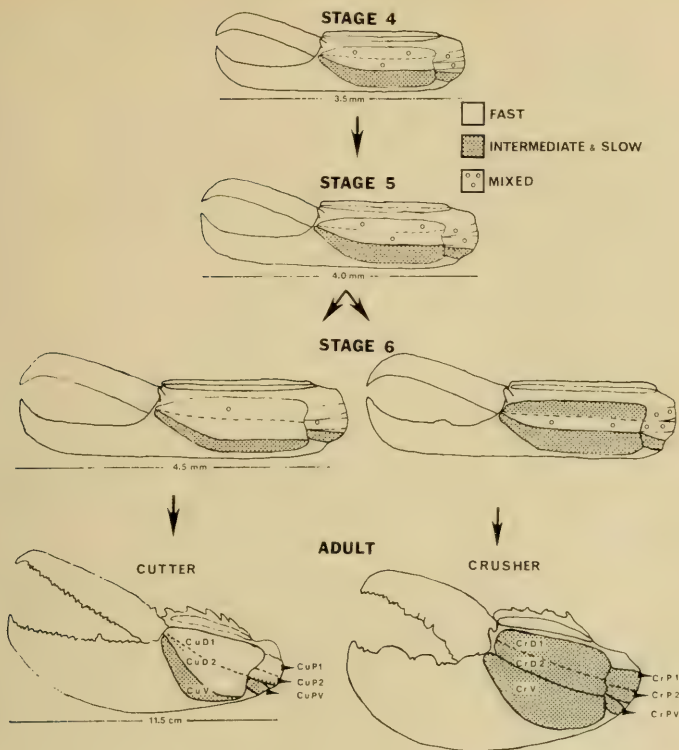


FIGURE 4. Medial view of lobster claw closer muscle in stages 4-6. Sequence of development is illustrated for the groups and their fiber composition. Number of symbols for mixed population of fibers (0) is proportional to the amount of slow fibers present in group (refer to the areas where they occur in Figs. 5-6).

slow fiber compositions in the dorsal groups are calculated, the proportion of one to the other became nearly equal (Table II). The percentage of fast fibers in these dorsal groups was 47%; the remaining fibers were either intermediate (18%) or slow type (35%).

The fast fibers within group C-D were apparently concentrated in one of its subunits. The percentage of fast fibers was generally higher in C-D2 (avg = 54%) than in C-D1 (avg = 38%). These values are significantly different at $P = 0.10$ (note fiber distribution between these subunits in Fig. 5).

IV STAGE LOBSTER CLAWS

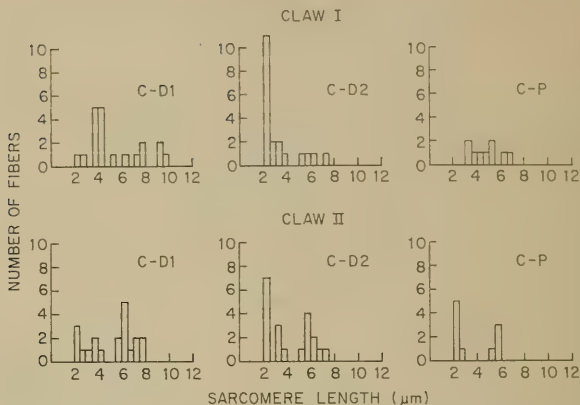


FIGURE 5. Frequency histograms of sarcomere lengths of fibers in the dorsal groups of the paired closer muscles in a stage 4 animal. These were the groups containing mixed populations of fast, intermediate and slow muscle fibers. The ventral groups (not shown) never had fast fibers.

Stage 5. At this stage the claws remain symmetrical. Likewise, the shapes of the groups in the closer muscle were congruent between claws throughout this stage (Fig. 4). The dorsal groups in the paired claws, excepting Claw I from a late stage 5 animal (discussed below), maintained populations of fibers with short, intermediate, and long sarcomere lengths. The localization of fast fibers to the subunit C-D2 noted in stage 4 was increasingly evident at this stage. This subunit contained more than twice as many short sarcomere fibers (avg = 86%) as subunit C-D1. The ventral groups of every closer muscle lacked fibers with short sarcomere lengths. The population of intermediate sarcomere length fibers decreased in these groups (avg = 8%). The average sarcomere length of fast fibers from this stage was essentially the same ($2.4 \mu\text{m}$) as in stage 4. That of the slow fibers exhibited a slight increase in length (avg = $7.3 \mu\text{m}$).

In the early fifth stage the distribution of fiber types was reported to be unchanged from that of the fourth, while a large increase in fast fiber composition in one claw was indicated in a late fifth stage animal (Govind and Lang, 1978). These observations were confirmed in the present study. The closer muscles from the paired claws of the mid-fifth stage animals were not significantly different in fiber type. However, the closer muscle of Claw I from a late fifth stage animal showed a substantial increase in the short sarcomere fiber population of the dorsal groups (Table I and II). This population was significantly different from that of Claw II.

Stage 6. When the animal molted to this stage, significant changes took place

TABLE II

Distribution by group of fiber types in claw closer muscles of postlarval lobsters.

Stage	Dorsal Groups (C-D, C-P)						Ventral Groups (C-V, C-PV)					
	Claw I/Cutter			Claw II/Crusher			Claw I/Cutter			Claw II/Crusher		
	<4 μm	4-6 μm	>6 μm	<4 μm	4-6 μm	>6 μm	<4 μm	4-6 μm	>6 μm	<4 μm	4-6 μm	>6 μm
4	50%	4%	46%	48%	12%	40%	0%	0%	100%	0%	20%	80%
4	37	12	51	45	34	21	0	27	73	0	22	78
4	52	24	24	48	22	30	0	33	67	0	27	73
5	66	20	14	44	8	48	0	10	90	0	3	97
5	70	0	30	60	2	38	0	13	87	0	0	100
5 (late)	100	0	0	50	13	37	0	20	80	0	3	97
6	94	4	2	31	4	65	0	40	60	0	0	100
6	94	6	0	30	0	70	0	7	93	0	10	90
6	90	0	10	34	0	66	0	0	100	0	7	93

in the external morphology of the claws. These are discussed in the next section. Changes were also evident in the structure of the closer muscle groups and in their fiber composition. The dorsal group (CuD) of the closer muscle in the cutter became larger than the ventral group CuV (Fig. 4). These groups in the crusher did not exhibit any noticeable changes in structure.

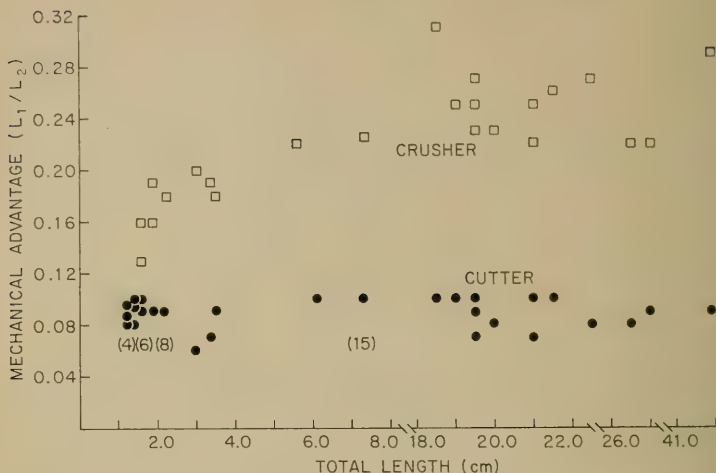
The distribution of fiber types in the muscle groups clearly reflected the differentiation of each claw (Fig. 6; Table II). In the dorsal groups of the cutter,

VI STAGE LOBSTER CLAWS



FIGURE 6. Frequency histograms of sarcomere lengths of fibers in the dorsal groups of the paired closer muscles in a stage 6 animal. Note virtual disappearance of slow muscle in CuD-CuP. Though fast muscle in CrD-CrP was decreasing as a whole, a substantial amount (50% of the sub-unit) was still present in CrD2.

MECHANICAL ADVANTAGE OF LOBSTER CLAWS



(M.A.) of each claw in the early forms. Indeed this was the case (Fig. 7). However, at stage 6 the values diverged for the two claws. One claw, the presumptive cutter, remained unchanged in its mechanical advantage ($\text{avg} = 0.10$). In fact, the value for this claw was virtually the same in all sizes of lobsters measured (M.A. range 0.06–0.10). In contrast, the contralateral claw (presumptive crusher) exhibited an increase in value to 47% more than that of the other. At each successive stage, the difference became greater. By stage 8, the presumptive crusher claw had attained a value of 0.18, about twice the value of the other claw. This trend continued, but at a slower rate, until the adult value was reached ($\text{avg M.A.} = 0.25$).

The major factor contributing to these differences in M.A. was the size of L_1 . The cutter L_1 was always shorter than the crusher L_1 . The value of L_2 in the cutter claw, conversely, became greater than the crusher's at each molt after stage 6. This served to complement the differences of L_1 .

DISCUSSION

The claws of the lobster are symmetrical in the larval stages (1–3) and in the early postlarval stages (4–5) (Herrick, 1911). The distribution of fast and slow fibers in the closer muscles is also virtually identical between the paired claws (Lang, Govind, and She, 1977; Govind and Lang, 1978). Not until the late fifth stage do changes in fiber-type composition appear. Physical differences between the claws occur during the subsequent stage. Once this stage is attained, both claws are asymmetrical in physical appearance as well as in muscle fiber populations in their respective closer muscles. The closer muscle of the adult cutter claw maintains fast and slow fiber types; that of the crusher claw contains only slow muscle.

It is clear that the changes in muscle fiber composition which occur during growth are limited to particular regions of the claws. In the younger lobsters, the ventral groups, C–V and C–PV, of the paired claws are basically homogeneous, never containing short sarcomere fibers. Apparently, they retain the same fiber type throughout the life span of the animal. However, in the dorsal groups, C–D and C–P, a different sequence of events leads to development of their adult pattern. In early stages, the dorsal groups of both claws contain short sarcomere fibers almost equal in proportion to that of the intermediate and long sarcomere fibers. By late stage 5, the dorsal groups of the presumptive cutter have lost most of the intermediate or long sarcomere fibers. Conversely, not until the subsequent stage do the homologous groups in the contralateral claw commence to lose the short sarcomere fibers. The two processes continue as the lobster matures until the adult stage is reached when every group is essentially homogeneous in regard to fiber type.

These two trends, increase and decrease of short sarcomere fibers in the cutter and crusher respectively, apparently occur at different rates. At stage 6, after claw differentiation, substantial numbers of short sarcomere fibers remain in CrD, whereas the same group in the cutter is almost devoid of intermediate and long sarcomere fibers. This is particularly noticeable in CrD2, in which over half of the fibers have short sarcomeres, although its adjacent subunit CrD1 contains less than

10% of such fibers. It is probably in subunit CrD2 that fast fibers observed in the crusher claw of later stages are located (Goudey and Lang, 1974; Govind and Lang, 1978). These results parallel the observations of Emmel (1908) that during stage 4 the claws are equally able to become cutter or crusher. If a claw is removed at this time, the remaining claw invariably becomes a crusher, regardless of the side. Even in early stage 5 (1-2 days after molting) this can occur. After this critical period, claw type is fixed and, when a claw is removed, the remaining claw has an equal probability to become a crusher or a cutter (Emmel, 1908; Lang, Govind, and Costello, 1978).

The functional morphology of claws has been studied in adult forms of several crustaceans (Warner and Jones, 1976; Vermeij, 1977). However, the development in the younger animals that proceeds to form the adult structures has apparently not been investigated. In examining the functional morphology of the younger forms prior to and after specialization, the development of the mechanical advantage has been shown here to be closely timed with the internal differentiation of closer muscle structure and fiber content to form dimorphic claws. The cheliped of the lobster which develops into the cutter claw becomes specialized, in several different ways, for activity of a phasic nature. The quick response enables it to capture prey and to play specific roles in agonistic behavior and defense (Scrivenner, 1971; Lang, Govind, Costello, and Greene, 1977). Indeed, it is capable of closing in less than 20 msec (Govind and Lang, 1974). However, by having characteristics which permit rapid closure, it has sacrificed the ability for strong, sustained contractions (Jahromi and Atwood, 1971). For instance, the mechanical advantage of the claw is low and does not change during growth. This low mechanical advantage is associated with a fast closing time (Alexander, 1968). Likewise, the cutter closer muscle has a large proportion of fast fibers. However, if the closer muscle lacked slow fibers entirely, the fast fibers would be responsible not only for closing the claw each time, but for maintaining closure if required. Successive contractions from these fast muscle fibers decrease rapidly in amplitude, probably as a result of synaptic fatigue (Jahromi and Atwood, 1971; Govind and Lang, 1974). After several contractions to close the dactyl or after a maintained contraction to keep it closed, the system would be fatigued, rendering the claw temporarily useless. Yet the cutter claw in intact lobsters can remain closed and can hold objects for several minutes (unpublished observation). Undoubtedly, it is the slow fibers, which comprise almost 20% of the cutter closer muscle, which accomplish this.

It seems clear that the crusher claw functions primarily in a tonic fashion. During the early juvenile stages, morphology of this claw changes such that it develops a large mechanical advantage, associated with maximal force development (Alexander, 1968). Later, the crusher closer muscle will be composed entirely of slow type fibers. Thus it has become highly specialized for developing a large amount of tension and for maintaining this tension during prolonged periods.

The existence of the bundles, dividing a single muscle into several subunits, leads to the inevitable question of their significance. Since the cutter closer muscle bundles (Fig. 3) are essentially homogeneous in regard to each fiber type, they might act as integral motor units possessing characteristics of the predominant fiber type. In other words, CuD could act as a phasic motor unit while CuV

would be used primarily as a tonic motor unit. The same would apply to CuP and CuPV, respectively. Preliminary results on mapping of the pattern of innervation by the motor axons suggests that this may be the case. In the crusher, all the bundles would function as mainly tonic motor units, working in conjunction with each other to develop the necessary force with the characteristic resistance to fatigue.

A further structural difference between the claws can be seen upon examination of the apodemes ("tendons"). In particular, the apodeme structure of the crusher closer muscle reflects the larger forces produced in this claw. The distal aspect where it attaches to the dactyl has a shelf which is wider and thicker than that in the cutter. This provides greater strength and also allows the insertion of additional fibers. Moreover, the apodeme as a whole is thicker in the crusher. The overall increased thickness possibly functions to withstand the greater force produced by the tonic muscle fibers as they contract.

One of the questions raised, but not answered, in the present study is in regard to the changes occurring in the muscle fiber composition of the dorsal groups of the closer muscle. That is, what happens to the short sarcomere fibers in the crusher claw and the long sarcomere fibers in the cutter? Do they degenerate completely or become transformed into the opposite type fiber? Any answer will have to account for the fact that in the cutter, long sarcomere fibers in the dorsal groups disappear while these same type fibers in the adjacent ventral groups remain unchanged, persisting through adult life. It seems likely that fibers with intermediate sarcomere lengths can become longer sarcomere slow fibers as it has been shown that sarcomere lengths increase within this range during growth (Govind, She, and Lang, 1977). This possibility was pointed out in an earlier paper of this series (Lang, Govind, and She, 1977). However, whether short sarcomere ($2-4\text{ }\mu\text{m}$) fibers can transform directly into long sarcomere fibers ($6-12\text{ }\mu\text{m}$) and vice versa is uncertain. The alternative hypothesis, of course, is that the fibers are lost through degeneration and are replaced by new fibers, formed *de novo*. Although we have little direct evidence bearing on this matter from the present study, we have not observed degenerating fibers during the stages when the fiber populations are changing. Furthermore, it has been demonstrated that nearly complete transformation to the opposite type can occur in vertebrate fast or slow skeletal muscle (Harris, 1974; Gutmann and Carlson, 1975). Whether this can occur in crustaceans will require further investigations.

In the vertebrates a motor unit consists of a motor neuron and the muscle fibers it innervates. Crustaceans, on the other hand, possess neuromuscular systems more parsimonious in nature. One or a few motor axons innervate an entire muscle. Such a situation might severely limit the flexibility of the motor system unless there are mechanisms providing for a wide range of contraction strengths and speeds. One such mechanism has been the formation of synapses with different degrees of facilitation, permitting recruitment of muscle fibers in a frequency dependent manner (Atwood, 1972). The groups examined in this paper might be another means for achieving flexibility of muscle function. They could represent an example of specialization to develop several motor units within a single muscle. These encompass a large number of fibers of similar type (sarcomere length). Together, the units can partition their activity and function in accordance with

innervation, fiber type, and physiological characteristics to control the motor activity of a structure.

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SUMMARY

1. The changes in functional morphology of the claws were examined during growth of the lobster by comparing claw structure (mechanical advantage) with muscle fiber properties in each of the closer muscles.

2. In the first two juvenile stages (stages +5) the claws and the closer muscles are essentially identical to one another. During late stage 5 or in stage 6, claw asymmetry becomes apparent in terms of the complement of muscle fiber types found in the closer muscles. During stage 6, claw morphology becomes asymmetric.

3. The fibers of the closer muscles in both claws at all stages were organized into distinct groups. In adult claws each group contained an homogeneous population of fast or slow muscle fibers.

4. In the adult cutter claw closer muscle, dorsal groups contained fast muscle while ventral groups had only slow muscle. On a dry weight basis, the cutter claw closer muscle consisted of 81% fast muscle and 19% slow muscle. The closer muscle of the crusher claw contains all slow muscle.

5. In postlarval animals (stages 4-6), the dorsal groups of the closer muscles in both claws contained a mixed population of fibers. Commencing at late stage 5, these groups in the presumptive cutter claw began to lose intermediate and slow fibers. At stage 6 the homologous groups in the presumptive crusher claw began to lose their fast fibers. The ventral groups in both claws never contained fast fibers.

6. At stages 4-5 the mechanical advantage (M.A.) of the paired claws was the same. During maturation the M.A. of the crusher claw increased until in the adult it averaged 2.8 times that of the cutter claw, illustrating the correlation of claw structure to fiber type in its closer muscle.

7. Thus, discrete changes occur in the claws during growth of the lobster so that each possesses many specialized features, in regard to muscle fiber properties and claw mechanics, to provide the particular function of speed for the cutter claw and of strength for the crusher claw.

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LIGHT DEPENDENT ACTIVITY PATTERNS AMONG REEF CORALS: *MONTASTREA CAVERNOSA*

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The popular literature on coral reefs frequently notes the great number of reef animals which are only visible during the night. Among these nocturnal organisms are the reef corals. Although their skeletons are always in evidence, the majority of reef corals can only be seen in a fully expanded condition during the night. The generalized activity cycle for scleractinians is one of daytime contraction and nocturnal expansion and feeding. In fact, not all scleractinians follow this pattern. A number of species expand during the day and there are some species which expand only during the day (Yonge, 1930; Kawaguti, 1937; Abe, 1938, 1939; Porter, 1974; Sweeney, 1976).

Expansion activity cycles are also known to vary within species. On the Caribbean coast of Panama, colonies of *Montastrea cavernosa* can be divided into two morphs on the basis of their activity cycles and polyp morphology (Lehman and Porter, 1973; Lasker, 1976, 1978). Polyps of the diurnal morph of *M. cavernosa* are expanded both day and night, while those of the nocturnal morph expand only at night. The presence of these differing activity cycles within a single species provides a unique opportunity to assess the relative importance of the different proximal and causal factors which control activity cycles among corals.

At the operational level, diel patterns of both nocturnal and diurnal expansion have been linked to a number of intrinsic and extrinsic factors. Innate circadian rhythms have been reported for both penatulids (Mori, 1960) and for corals (Sweeney, 1976). Other authors have related expansion among cnidarians to extrinsic factors like light (Hargitt, 1907; Abe, 1939; Pearse, 1974; Gladfelter, 1975; Krezoski, 1977), oxygen concentration (Abe, 1939; Brafield and Chapman, 1965, 1967; Sassaman and Mangum, 1972; Wells, Wells and Van der Walker, 1973) and ammonium concentration (Kawaguti, 1937).

Causally, nighttime expansion has traditionally been related to the increased availability of prey (zooplankton) during the evening. Indeed, zooplankton are more abundant in reef waters at night (Glynn, 1973; Porter, Porter and Batac-Catalan, 1977). However, a variety of detrital prey items are available during the day and corals can and do feed on these particles (Price and Lewis, 1975).

Among coelenterates with symbiotic algae, daytime expansion has been related to the effects of expansion on photosynthesis. For instance, *Anthopleura elegantissima*, an anemone, has symbiotic individuals which are expanded only during the day and aposymbiotic individuals which are expanded both day and night (Pearse, 1974). On the other hand, the majority of reef corals, all of which have zooxanthellae, do not expand during the day or do so rarely.

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MATERIALS AND METHODS

In order to quantify *M. cavernosa*'s activity cycle, 125 colonies from three localities on Panama's Caribbean coast (Portobelo; Galeta Island; and Korbiski Reef, Islas San Blas) were labelled and monitored for daytime expansion and at irregular intervals over a two-year period. An additional 36 colonies which had been collected from Portobelo and Isla Salar were maintained in the sea tables at the Galeta Marine Lab and expansion data from these colonies were also collected. All daytime observations were made between 1000 and 1600 hr and the majority of observations were made between 1200 and 1500 hr. Individual colonies from Galeta were scored up to 81 times and those from Korbiski and Portobelo up to 24 and 18 times, respectively. Quantitative nighttime observations were made of the Korbiski reef colonies (5–11 observations per colony) and of the sea table colonies (13–25 observations per colony). Qualitative nighttime observations of the Galeta colonies were also made. All nighttime observations were made between 2000 to 2400 hr.

Polyp expansion was scored for each of the colonies on a scale ranging from 0, complete contraction, to 4, full expansion. The scores refer to individual polyps and the values presented for whole colonies are field estimates of the average degree of polyp expansion occurring on a single colony. The five classes are illustrated in Figure 1. An expansion state of 0 indicates complete contraction of the polyp such that neither tentacles nor oral disk are in view. When the polyp expands to the point at which its tentacles, though still contracted, are visible it is assigned a score of 1. Further expansion bringing the oral disk into sight is indicative of a score of 2. A score of 3 indicates expansion of the outer whorl of tentacles and a score of 4 indicates complete expansion of all of the tentacles. The presence or absence of sweeper tentacles (Den-Hartog, 1977) is not considered in the scale. The scale is also independent of tentacle length and other characteristics which differ systematically between the two morphs. Adjustments for factors like tentacle length and individual "quirks" in behavior require an experienced observer for consistent observations. However, an inexperienced observer can readily distinguish expansion states two units apart.

In addition to having their expansion behavior monitored, 18 sea table colonies were used for two sets of experiments. The first of these, the altered light experiments, tested the sensitivity of the activity cycle to light. During these experiments colonies were subjected to nighttime lighting and to mid-day darkness. In the nighttime lighting experiments colonies were exposed to 120 min of low intensity light ($9 \mu\text{E}\cdot\text{m}^{-2} \text{ sec}^{-1}$ at the top of the sea table) provided by an outdoor floodlight. The period of artificial illumination extended from 2100 to 2300 hr on each of five evenings. Daytime darkness was achieved by covering the sea tables with black polyethylene sheets for a two hour period between 1100 and 1300 hr. The daytime darkening experiment was also replicated five times for each colony. Expansion states of the colonies were monitored at 30 min intervals starting 90 min before the altered light condition and continuing to 90 min past the light treatment. Noontime light levels in the sea tables were characteristically between 75 and $100 \mu\text{E}\cdot\text{m}^{-2} \text{ sec}^{-1}$. Colonies were kept in flowing aerated seawater ($27\text{--}29^\circ \text{C}$) at all times during the experiments.

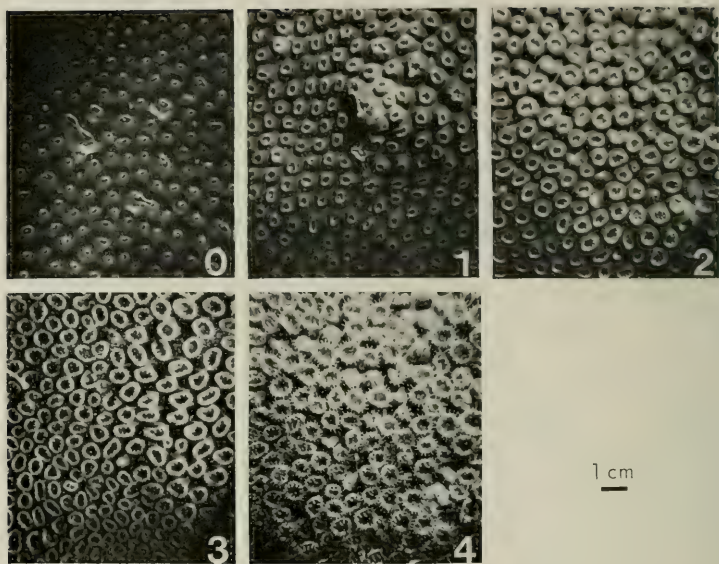


FIGURE 1. Expansion states of *M. cavernosa*. Photographs of the five expansion states of *M. cavernosa* colonies. The scores in the lower right hand corner of each photograph are visual averages for the entire field of view. Note the variation between individual polyps in expansion state 2. See text for complete description of the expansion states.

The second set of experiments involved the treatment of diurnal morph colonies with the photosynthetic inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea). Colonies used in the DCMU experiments were transferred from the sea tables to a 10 liter aquarium several hours prior to the experiment and were kept in flowing aerated sea water until the start of the experiment at which time water flow was discontinued and DCMU added. One liter of DCMU saturated sea water was added to the aquarium to yield a theoretical concentration of 10^{-4} M. Difficulties in dissolving the DCMU suggests a somewhat lower realized concentration (see Vandermuelen, Davis and Muscatine, 1972). Expansion was monitored at 30 min intervals starting 90 min prior to the DCMU addition and continuing for 120 min into the treatment. Light levels during the experiments were the same as those for the daytime darkening experiments.

RESULTS

Activity cycles of the morphs

The observations of daytime expansion for each of the colonies from three of the localities were subjected to a Q-mode cluster analysis using a quantified

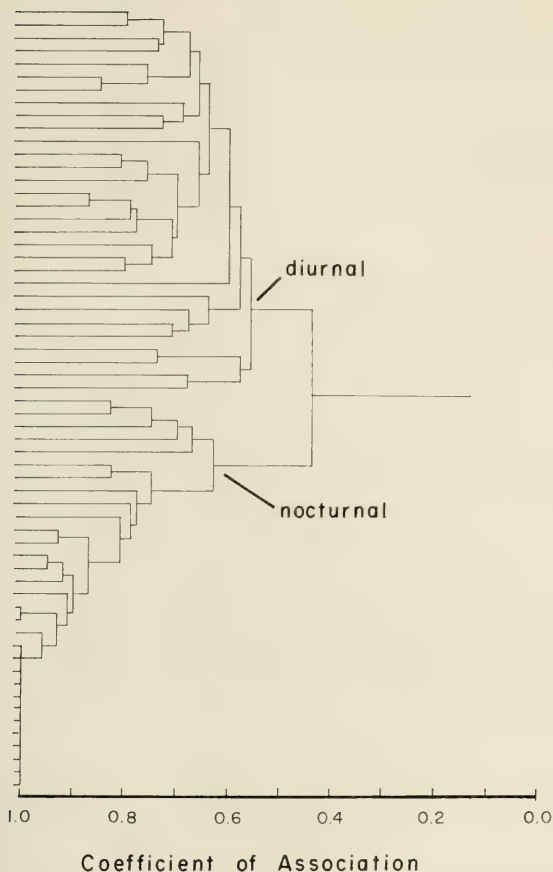


FIGURE 2. Cluster analysis of colonies on Korbiski reef based on their daytime expansion patterns. Two clusters corresponding to the two morphs are distinctly visible.

Jacard's coefficient of similarity (Sepkoski, 1974). The fourth data set, Portobelo, was not clustered due to an insufficient number of observations. The clustering procedure broke the colonies into groups with similar behaviors. A dendrogram relating the similarities in expansion behavior of the Korbiski colonies is presented

TABLE I

Frequency of expansion of diurnal and nocturnal morph colonies from Korbiski Reef, Islas San Blas.

		Expansion state					Number of total observations
		0	1	2	3	4	
Diurnal Morph	day	0.20	0.24	0.32	0.22	0.02	551
(28 colonies)	night	0.03	0.04	0.04	0.12	0.77	278
Nocturnal Morph	day	0.81	0.12	0.05	0.02	0.00	605
(33 colonies)	night	0.03	0.03	0.03	0.14	0.77	238

in Figure 2. The colonies in this dendrogram and in the dendrograms for the other localities can readily be divided into two classes. These correspond to colonies which were not regularly expanded and those which were. This classification agrees, of course, with the definitions of the morphs and demonstrates that the dichotomous view of the activity cycles accurately reflects the natural distribution of behavioral patterns within *M. cavernosa*. Both the mean and modal expansion state of the colonies in Figure 2 mirror the two-morph classification, but a classification based on the presence or absence of a non-zero mode appears to most accurately reflect the results of the cluster analysis. When the colonies were separated according to their modal expansion state 92% of the classifications were in agreement with the cluster analysis.

The frequency of expansion for the two morphs during both day and night are presented in Table I. There are radical differences in expansion between the diurnal morph and the nocturnal morph during the day, when the nocturnal morph is almost always contracted. At night, however, when the nocturnal morph expands, there are no significant differences in the expansion states of the two morphs. It is of interest to note that there are significant differences (χ^2 test, $P < 0.001$) in the expansion state of the diurnal morph between the day and night. During the day expansion states 2 and 3 are most common while at night state 4 is most common.

The clustering pattern in Figure 2 and those of the colonies from the other localities, despite their dichotomous nature, also suggest that a variety of behavioral patterns can be found within each of the two classes. In the Korbiski dendrogram (Fig. 2) a considerable spread of similarities can be found within the nocturnal class, and within both clusters similarities as low as 0.60 can be found. Dissimilarity within clusters can be attributed to the presence of colonies with intermediate behaviors. During the day the archetypical diurnal morph colony is fully expanded all of the time and the archetypical nocturnal morph colony is fully contracted. However, in nature many intermediates occur. Diurnal morph intermediates are expanded during the day but are not always fully expanded, and nocturnal morph intermediates are sometimes expanded during the day, but usually at very low levels of expansion. Intermediates provide a substantial middle ground in the nocturnal/diurnal classifications.

Environmental effects also play a role in blurring the distinction between the morphs. The cluster analysis of the Galeta Island colonies suggested that the

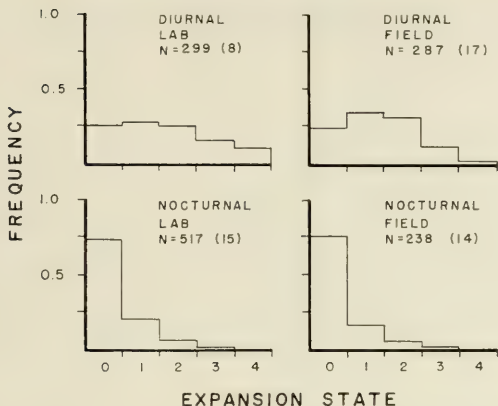


FIGURE 3. Effects of the laboratory environment on daytime expansion behavior. Frequency distributions of expansion behavior are presented for colonies from Portobelo, which were observed in the field, and for a second set of Portobelo colonies which were maintained in sea tables at the Galeta Marine Laboratory. Separate distributions are presented for the two morphs. N indicates the total number of observations and the value in parentheses indicates the number of colonies observed.

diurnal morph colonies could be divided into two subgroups which had a level of association of 0.64. Those subgroups corresponded very strongly with the distribution of the colonies. *M. cavernosa* colonies at Galeta Island occur predominantly in two localities each of which is subject to differing amounts of wave action. The colonies from these different areas cluster together. Although the proximity of the corals to each other and their similar appearances suggests a strong degree of relationship within areas, the existence of environmental modification is a distinct possibility.

A second line of evidence for an environmental effect is to be found in a comparison of the daytime observations of Portobelo colonies from the field and in the laboratory. The labelled colonies in the field and those collected for sea table observations were both chosen in an essentially random fashion, and should within sampling error have similar behavioral patterns. Among the nocturnal colonies, which are hardly ever expanded during the day, this is indeed the case. However, the expansion patterns of the field and lab diurnal morph colonies differ significantly (χ^2 test, $P < 0.001$) (Fig. 3). Laboratory colonies expanded as frequently as the field colonies, but when expanded the laboratory colonies were more fully expanded. This can be seen in the relative proportions of expansion scores of 1 and 2 versus those of 3 and 4 in Figure 3. While the differences between the laboratory and field environments do not affect daytime expansion itself, they do apparently inhibit the degree of expansion.

During the night, the laboratory environment inhibits expansion. Korbiski

Reef colonies observed in the field were fully expanded during the night (Table I) and qualitative observations at Galeta Island and at other localities indicate that in the field almost all colonies of *M. cavernosa* are fully expanded at night. In the laboratory, however, fewer colonies of both morphs were expanded, and colonies were frequently only partially expanded. In the field there were no nighttime differences between the morphs, but at night in the laboratory the diurnal morph was less frequently expanded than the nocturnal morph (χ^2 test, $P < 0.001$). This indicates that the diurnal morph was more strongly affected by the laboratory environment.

The presence of the intermediates and of environmental effects makes it impossible to present a statistically homogeneous characterization of the behaviors of the two morphs. As seen in Figure 3, distributions from different environments differ from each other and in other cases colonies within the same locality and morph can be found to have significantly different expansion patterns. It is possible, however, to describe generalized patterns for the morphs. The frequency distributions of daytime expansion behavior of four representative colonies are presented in Figure 4.

Effects of altered light regimes

The results of altering the daily light cycle are illustrated in Figures 5 and 6. As can be seen, the experiments demonstrate rather dramatically that the nocturnal morph cues its contraction to the presence of light. In the darkening experiment

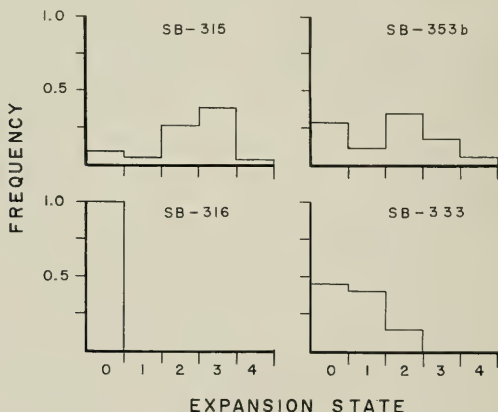


FIGURE 4. Distribution of expansion states occurring within the morphs of *M. cavernosa*. Frequency distributions are presented for the daytime expansion behaviors of colonies representative of the end member and intermediate conditions of the diurnal (SB-315 and SB-353b) and nocturnal (SB-316 and SB-333) morphs.

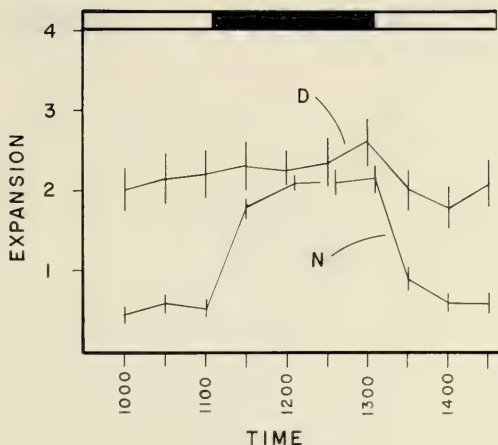


FIGURE 5. Effects of darkness on daytime expansion. Means with standard errors are plotted for the means of 5 replicate tests of each of 9 diurnal (D) and 25 nocturnal (N) colonies which were shaded from all light for a 120 min period at midday. Periods of darkness and light are indicated by the shaded bar at the top of the graph.

(Fig. 5) there is a marked increase in expansion of the nocturnal morph immediately after the colonies are shaded. Observations indicate that this expansion took place primarily during the first 30 min of darkness but continued through the following 30 min period. Within minutes after reexposure to shaded sunlight the nocturnal morph colonies began to contract. Most of the contraction occurred within the first 10 min of light, but the trend continued the first hour, at which time the colonies reached their daytime level of expansion.

The nocturnal morph colonies exhibited the same light response in the evening experiments (Fig. 6). In those experiments lighting led to a decrease in expansion. The light levels, which approximated dawn lighting, did not, however, cause the nocturnal morph colonies to return to full daytime contraction. After the lights were extinguished, the contracted colonies slowly expanded to the pre-lighting condition.

In both experiments the expansion of the diurnal morph remained almost constant. However, there were distinct, though small, light effects on the diurnal morph as well as on the nocturnal. In the daytime darkening experiments a slight increase in expansion occurred in the dark condition. That increase was reversed when the colonies were returned to natural lighting. Similarly, there was a decrease in expansion during the light period of the nighttime experiments and that too was reversed when the colonies were returned to natural lighting. It appears that both morphs can sense and react to relatively low light intensities. The effect of light on the diurnal morph, however, causes only a slight decrease in polyp

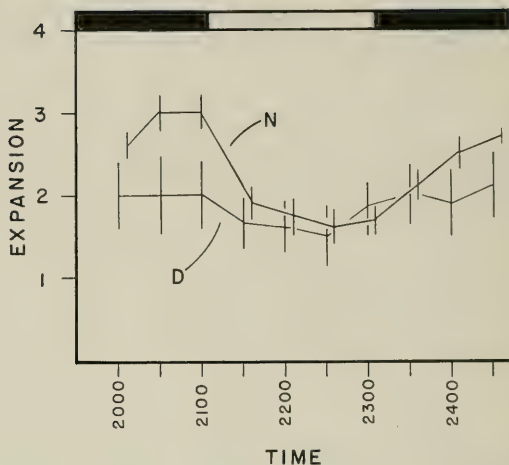


FIGURE 6. Effects of lighting on nighttime expansion. Means with standard errors are plotted for the means of 5 replicate tests of each of 9 diurnal (D) and 25 nocturnal (N) colonies which were exposed to 120 min of low level light during the night. Periods of darkness and light are indicated by the shaded bar at the top of the graph.

expansion while in the nocturnal morph the light induced contraction is almost complete.

Effects of DCMU

The results of the DCMU experiments are presented in Figure 7. As is evident, the DCMU has no effect on expansion. Shick and Brown (1977) have argued that photosynthetic products like oxygen may mediate expansion in symbiotic coelenterates. In other corals DCMU is known to inhibit photosynthesis (Vandermuelen, Davis, and Muscatine, 1972), and respirometric measurement of a *M. cavernosa* colony treated with DCMU resulted in a 94% ($\pm 6\%$) decrease in gross primary productivity. The failure of DCMU to affect expansion despite its inhibition of photosynthesis suggests that, under laboratory conditions, photosynthetic products are not important in the proximal control of daytime expansion.

DISCUSSION

The experimental results indicate that under normal conditions the addition of DCMU and the subsequent inhibition of photosynthesis does not affect daytime expansion. This result, in concert with the altered light experiments, suggests that at the proximal level expansion behavior is controlled by direct light sensing and not by the sensing of a photosynthetic product.

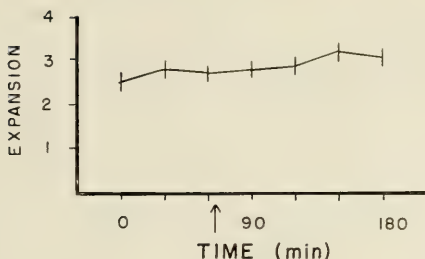


FIGURE 7. Effects of DCMU on daytime expansion. Means and standard errors for the behavior of 15 diurnal morph colonies which were exposed to DCMU. The colonies were kept in aerated sea water in shaded sunlight throughout the experiment. The DCMU was added at the time marked by the arrow.

Although the production of photosynthetic products does not control expansion *per se*, the zooxanthellae and presumably their products can be shown to be of major importance to daytime expansion. *M. cavernosa*, like other corals, expels its zooxanthellae when subjected to extremes of salinity, temperature, light and other physical parameters (see, for instance, Goreau, 1964). Colonies usually do not expel all of their zooxanthellae, but they do take on a white "bleached" appearance and regeneration of the zooxanthellae population, if it occurs, requires several months. In December 1976 one small region of a colony which was being monitored lost its zooxanthellae and developed a distinct bleached appearance

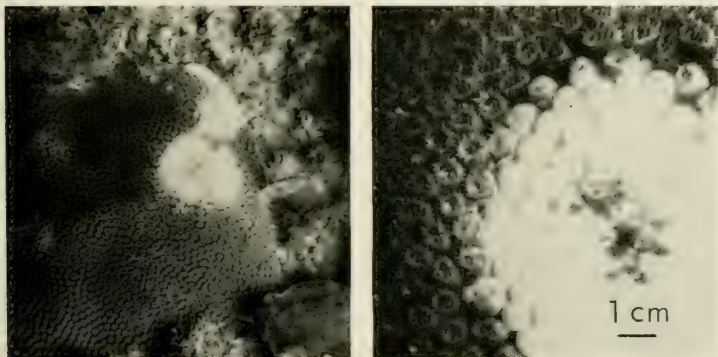


FIGURE 8. Partial bleaching of a *M. cavernosa* colony. The colony, shown on the left, is approximately 1 m in height and was found at 8 m depth on the reef at Galeta Island. The bleached area, shown in closeup on the right, does not have expanded polyps. Note that lightly pigmented polyps exhibit partial expansion.

TABLE II

Gross primary productivity. Mean gross primary productivity ($\pm$ one standard error) for expanded and contracted colonies of the two morphs of *Montastrea cavernosa*.

Light Intensity (μ E-m ⁻² -sec ⁻¹)	Gross Primary Productivity (μ g O ₂ -cm ⁻² hr ⁻¹)		
	Diurnal Morph (N = 8)		Nocturnal Morph (N = 8)
	Expanded	Contracted	Contracted
25	*22.36 (2.01)	17.64 (1.20)	9.78 (1.59)
93		28.07 (2.53)	21.63 (1.42)
1890		41.75 (2.78)	31.48 (2.62)

* N = 10.

(Fig. 8). The bleaching episode occurred during a nine day period of heavy wave action during which observations could not be made. After the appearance of the bleached area, the pigmented portion of the colony continued to expand during the day as it always had. The bleached section, however, no longer expanded during the day, but did expand at night. As the bleached area slowly developed some pigmentation, it also began to exhibit some daytime expansion. The degree of daytime expansion increased as the degree of pigmentation increased. The colony continued to expand nocturnally throughout this time period, and in all other respects behaved normally. The loss of the daytime expansion appears, therefore, to have been integrally linked to the absence of zooxanthellae. This same sequence has been observed in other colonies frequently involving areas as small as a single polyp. In all of these cases the polyps appeared otherwise healthy but nighttime expansion data are available only for the single case. The presence of zooxanthellae apparently has a definite effect on *M. cavernosa*'s expansion behavior even if the zooxanthellae do not control expansion behavior on a day to day basis.

The short-term effect of the zooxanthellae as deduced from the DCMU experiments and the longer term effects implied by the behavior of partially bleached colonies are, at first inspection, contradictory. Pearse (1974) described a similar situation among individuals of *A. elegantissima*. She noted that bleached individuals retained a diurnal expansion rhythm, unlike the naturally occurring aposymbionts, and she suggested that the retained behavior had been entrained in the previously symbiotic individuals. Her hypothesis also explains the failure of DCMU to have any effect on *M. cavernosa*. If *M. cavernosa* colonies also develop an entrained daytime expansion response, the DCMU experiments would have occurred over too short a time period to detect any change in behavior. The maximum of nine days which was required for the colony described above to have lost its expansion behavior suggests that the time required for *M. cavernosa* to overcome the entrained response is relatively short.

The long-term importance of photosynthesis to the diurnal behavior is further supported by the distribution of daytime expansion over depth. Observation of 592 colonies in the Islas San Blas failed to produce a single observation of daytime expansion below 20 m (Lasker, 1977). Increased depth corresponds with de-

creased light and with reduced photosynthesis. This suggests that the diurnal behavior, if it is based on primary productivity, is sensitive not only to the presence or absence of photosynthesis but also to its absolute magnitude. A dependence on some minimal photosynthetic rate may explain the absence of the daytime expansion in the nocturnal morph which, though photosynthetic, is not as productive as the diurnal morph (Table II).

The final line of evidence supporting a model of photosynthetically controlled expansion behavior is to be found in the results of a series of colony transplant experiments which were started in February 1977. At that time seven colonies were transplanted from 2 to 14 m and vice versa. Each of the colonies transplanted was one of a sib pair. The sib, a morphologically identical colony occurring on the same ancestral corallum but now separated by a dead or overgrown area, was left at its original depth. The expansion behaviors of the sibs were compared at monthly intervals over the following six months and again after 18 months. The colonies transplanted to shallow water showed no change in behavior, but of the colonies transplanted to deep water, three showed a significant decrease in expansion behavior (randomization test, (Siegel, 1956) $P < 0.05$). Possibly the deep-water nocturnal morph colonies which naturally have lower rates of photosynthesis (Lasker, 1978 and Table II) could not develop daytime expansion even in a high light environment. On the other hand, the shallow water diurnal morph colonies, when deprived of sufficient light, ceased daytime expansion.

Other inter- and intraspecific comparisons of coelenterates with similar dichotomies of behavior have associated daytime expansion with the presence of symbionts (Kawaguti, 1937; Pearse, 1974; Gladfelter, 1975; Sebens and de Riemer, 1977). Although morphs of *M. cavernosa* are symbiotic, similar arguments may still be applied to their differing expansion behaviors.

The presence of expansion in symbiotic coelenterates has been previously attributed to an expansion related increase in photosynthesis (Lasker, 1977; Sebens and de Riemer, 1977). It has also been hypothesized that the lack of zooxanthellae and therefore, photosynthesis, makes daytime expansion disadvantageous for most species. This of course implies a "cost" of expansion. The principal argument for these hypothesized costs and benefits of expansion are the distributions of the zooxanthellae and the observed activity patterns themselves. Unfortunately, few data have been presented which directly document the existence of the costs and benefits.

One advantage of daytime expansion relates to the rate of photosynthesis occurring within the polyps. Much of the tissue of a polyp which is exposed to direct sunlight in the expanded state is shaded when the polyp is contracted. In *M. cavernosa* an increase in gross primary productivity associated with expansion has been observed (Table II, Mann Whitney U-test, $P < 0.05$). Similarly, Kanwisher and Wainwright (1967) and Svoboda (1978) have reported that oxygen production in soft corals is greatest when the colony is expanded.

The nature of the costs of expansion are less clear than the benefits. Energetic costs, daytime predation on expanded polyps, and nitrogen conservation have been considered by either Lasker (1977) or Sebens and de Riemer (1977). Sebens and de Riemer (1977) have noted that lack of evidence for differential predation on expanded polyps, and field observations made in the course of this study suggest

that species expanded during the day are no more heavily preyed upon than contracted species. However, the differences between expanded and contracted species may also include other types of anti-predator adaptations. Until more studies are carried out on the nature and extent of predation on corals it is impossible to dismiss a predator-related cost of expansion.

Daytime contraction may also be a nutrient conserving adaptation and Sebens and de Riemer (1977) have argued that nitrogen conservation may be the basis for the observed diel activity patterns of reef anthozoans. Again, however, there is no direct evidence for or against this hypothesis. In *M. cavernosa* the nocturnal morph, which is the superior zooplankton predator (Lasker, 1976), does not expand during the day while the diurnal morph does. If nitrogen is a limiting resource and if expansion does incur a nitrogen loss, one might predict that the diurnal morph, which is less adept at capturing prey and should therefore be under greater nitrogen stress, would be less likely to expand during the day. It may be the case that daytime expansion by the diurnal morph results in a net nitrogen gain through the capture of detrital matter.

Energetic costs of expansion have been noted (Pearse, 1974; Johannes and Tepley, 1974; Lasker, 1977; Sebens and de Riemer, 1977). Although expansion may involve several different costs, the only cost which has been measured to date is the increased respiration of expanded polyps. Increased respiration rates have been reported for the expanded polyps of *Anthopleura elegantissima* (Buchsbaum, 1968) and for *M. cavernosa* (Lasker, 1978). Johannes and Tepley (1974) presented data suggesting the same is true of *Porites lobata*. Similarly, respirometric increases related to expansion have been reported for several aposymbiotic species of coelenterate (Brafield and Chapman, 1965, 1967; Newell and Northcraft, 1967).

M. cavernosa, like other symbiotic coelenterates, exhibits characteristics demonstrating the existence of expansion related costs and benefits. In order to explain the differing behaviors of the two morphs it is necessary to find differences in the costs and/or benefits of expansion for the two morphs. Although both morphs of *M. cavernosa* have zooxanthellae, there are differences in the distribution of the zooxanthellae and in their photosynthetic capabilities, which affect the value of daytime expansion.

In colonies from shallow water (10 m or less) polyps of the diurnal morph have greater numbers of zooxanthellae than do those of the nocturnal morph (Lasker, 1978). A large proportion of the polyps' zooxanthellae are shaded in the contracted state (Lasker, 1978) and expansion increases gross photosynthesis.

Calculations based on measured photosynthetic rates of whole colonies and on zooxanthellae concentrations within the two morphs reveal that the increase in gross primary productivity (GPP) effected by expansion is greater for the diurnal morph than the nocturnal morph (Lasker, 1978). However, in the case of both morphs the expansion induced increase in respiration is even greater than the increase in GPP. The net effect of expansion is, therefore, an energetic cost to the colony.

The occurrence of expansion despite its net energetic cost suggests that the basis for the behavior, though triggered by light, is not purely photosynthetic. However, the photosynthetic differences between the two morphs and the presence

of expansion in the diurnal morph (the morph with the greater photosynthetic rates) suggests that some minimal photosynthetic input is necessary before non-photosynthetic advantages of expansion can be balanced against the energetic cost of expansion. Such a view is further supported by the absence of expansion in deeper waters where photosynthesis is reduced and by the absence of expansion in portions of colonies which have expelled their zooxanthellae. The differences in productivity and behavior of the morphs clearly indicates the importance of photosynthesis in the costs and benefits which ultimately control expansion behavior.

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SUMMARY

1. Colonies of *M. cavernosa* exhibit two separate types of activity cycle, one of nighttime expansion and one of night and daytime expansion. These activity cycles correspond to the nocturnal and diurnal morphs of this species.

2. At the operational level, light *per se* controls expansion behavior. Colonies are sensitive to light and react quickly to it. This sensitivity is not, on the short term, dependent on photosynthesis. The reaction to light of both morphs is one of contraction, but in the diurnal morph the contraction response is inhibited.

3. The inhibition of daytime contraction only occurs in the diurnal morph, which has a high rate of photosynthesis, and only occurs above 20 m (in Panama). Loss of zooxanthellae, or removal to deep water, both of which reduce photosynthesis, results in the loss of daytime expansion.

4. Although photosynthetically based light sensing is not involved in short term control of expansion behavior, colonies may measure their photosynthetic production, and if photosynthesis falls below some level, daytime expansion is halted. The sensing of conditions conducive to daytime expansion can occur within small areas of a single colony.

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ROLE OF AGGREGATION FACTOR AND CELL TYPE IN SPONGE CELL ADHESION

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Studies of sponge cell aggregation have provided some of the first insights into cell adhesion at the chemical level. Humphreys (1963) discovered that a substance, removed when cells from certain species were placed in calcium-and-magnesium-free sea water (CMF-SW), was essential for adhesion of cells from these sponges. Using *Microciona prolifera* and *Haliclona oculata*, these aggregation factors were species-specific in the promotion of aggregation. A mixture of CMF-dissociated cells of these two species formed separate aggregates which contained cells from only one species, and this aggregation occurred only if the respective factor had been added. Humphreys (1963) proposed that these factors were extracellular materials which served as linkages between cells. He particularly emphasized that the correspondence between the species-specificity of aggregation itself and the species-specificity of promotion of aggregation by the factor indicated that that factor served as an adhesive substance. Moscona (1968) supported this proposal with his demonstration that the factor was removed from solution when cells aggregated and that the factor promoted the adhesion of fixed cells.

Biochemical studies of Humphreys (1965) and Margoliash, Schenck, Hargie, Burokas, Richter, Barlow and Moscona (1965) indicated that the factors were particulate glycoproteins. Henkart, Humphreys and Humphreys (1973) showed that the factors were fibrous complexes of large size and had a sunburst configuration.

There have been several observations which have been interpreted as being in conflict with an adhesive role for aggregation factor. Sara, Liaci and Melone (1966) and Curtis (1970) obtained non-species-specific aggregation of sponges under certain conditions which Curtis interpreted as conflicting with such a role. In another study using a fresh water sponge, Curtis and Van De Vyver (1971) proposed that the aggregation effects observed with the factor were actually due to two enzymic substances produced by each species, rather than to adhesives. John, Campo, MacKenzie and Kemp (1971) presented a study of the role of two different cell types in the species specificity of aggregation of two marine sponges, in which they reported different specificities depending upon the types of cells present in the mixture. They interpreted their results in terms of an interaction of cell types and the presence of motility, and did not emphasize the role of an adhesive factor. Certain observations of MacLennan and Dodd (1967), which will be discussed further on in this paper, are particularly difficult to reconcile with the original idea of species-specific adhesive factors.

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To investigate the mechanism of action of the factors, an investigation of the aggregation of a set of sponges whose factors promote the adhesion of one another's cells was carried on. Using results of these experiments and a consideration of certain implications of Steinberg's differential adhesion hypothesis (Steinberg, 1963, 1964, 1970), a mechanism of action for the aggregation factor is proposed. The results indicate that cross-reactivity of factors is not inconsistent with an adhesive role; moreover, the data on cross reactivity has allowed a more detailed picture of the mechanism of action of the factor to be obtained.

The sponge factor hypothesis emphasizes the role of species-specificity in aggregation. However, it is well established (Wilson, 1907; Galtsoff, 1925) that a sponge is composed of cells of many types, and that these cells can reconstitute the characteristic structure of a sponge following disaggregation. Observations made here indicate that adhesive differences between different types of cells are important even during the early stages of aggregation, and affect the type and distribution of cells incorporated into aggregates. This finding raises new questions concerning the role of the factor in aggregation.

MATERIALS AND METHODS

Specimens of *Microciona prolifera* (*M*), *Halichondria bowyerbanki* (*B*), *Halichondria panicea* (*P*), and *Haliclona oculata* (*O*) were collected near Woods Hole, Massachusetts during the early summer and were used within two days. Mechanically dissociated cells were prepared by soaking pieces of sponge in Marine Biological Laboratory formula artificial sea water (MBL-SW) and then squeezing them through #25 bolting cloth. The resulting cell suspension was adjusted to approximately 60×10^6 cells per ml for the *Halichondria* species and to 30×10^6 cells per ml for *M* and *O*.

CMF-dissociated cells and aggregation factor were prepared according to Humphreys' (1963) method. Healthy sponge pieces were soaked in three changes of 0° C Marine Biological Laboratory formula calcium-magnesium-free artificial sea water (CMF-SW) and forced through bolting cloth. The resulting suspension was leached in CMF-SW for six hours at 4° C to remove the factor from the cells. The cells were precipitated in a clinical centrifuge and resuspended in MBL-SW at the above concentrations. The supernatant was spun at 17,000 G for 35 min and CaCl_2 was added to a concentration of 2 mM to the second supernatant, giving the aggregation factor.

Stained CMF-dissociated cells were prepared by adding approximately 1–20,000 part Nile Blue Sulfate (NBS) to the CMF-SW during the 6 hr leaching step.

Aggregation experiments were run in 25 ml Erlenmeyer flasks on a 80 rpm gyratory shaker at either 4° C or room temperature (22–26° C). Two ml of cell suspension were placed in each flask and factor (or MBL-SW) added to a total volume of 4 ml. Aggregates were scored visually and microscopically as to size and appearance at various times.

Petri dish cultures were kept in 35 mm plastic petri dishes (Falcon) at either 4° C or room temperature. The concentration of cells was adjusted to give a density suitable for microscopic examination.

For experiments requiring a single cell type, cells from a dissociated suspension

were separated according to their size using the technique of Miller and Phillips (1969). A stained suspension of CMF-dissociated cells in 0.5% sucrose in CMF-SW was introduced (from the bottom) into a cylindrical glass sedimentation chamber beneath a layer of CMF-SW. This cell-containing layer was followed by a 0.75 to 5% step gradient of sucrose in CMF-SW. The cells were allowed to sediment for three to four hours. Fractions were collected from the chamber outlet, concentrated by centrifugation and resuspended in MBL-SW.

RESULTS

CMF-dissociated cells

The following results apply to cells cultured at 4° C unless otherwise specified. Suspensions of *M* cells plus *M* factor showed strong aggregation compared to controls of *M* cells plus MBL-SW in all experiments at dilutions of up to 1/32 the original factor concentration. Aggregation was very advanced at two hours, at which time the aggregates were often several millimeters across. The controls had a very low but consistent level of aggregation, far lower than with the factor. The *B* factor gave inconsistent results in promoting the aggregation of *B* cells. In five out of ten trials, aggregation was enhanced over controls, but in only two of these cases was the aggregation extensive. This aggregation was slower than *M* aggregation. A suspension of *M* cells plus *B* factor showed no aggregation in seven out of nine trials and slight aggregation in the remaining two. *B* cells aggregated in seven out of eight trials with the *M* factor. These aggregates were typically looser and smaller than those of *M*, with a greater percentage of unaggregated cells. Dilution series showed aggregation of *B* by *M* factor to 1/8 the original concentration. The aggregates were tighter at lower factor concentrations and the cells looked healthier. Higher levels of *M* factor may thus be toxic for *B* and *P* cells.

A mixture of *M* and *B* cells with *M* factor gave intermingled aggregates which contained cells of both species (sometimes separate aggregates would form initially, but within three to four hours these would fuse and intermingle). Due to the uncertainty in distinguishing cells of the two species, reciprocal staining experiments were performed. CMF-dissociated, stained cells of one species were combined with unstained cells of the other species plus the *M* factor. Both such combinations yielded intermingled aggregates containing stained cells. Usually there was extensive intermingling but sometimes there were regions within an

TABLE I
Aggregation of CMF-dissociated cells in the presence of aggregation factors.

Cells	Factor			
	M	B	M + B	None
M	++++	+/-	++++	-
B	++	+/-	++	-
M + B	Intermingled	+/-	Intermingled	-

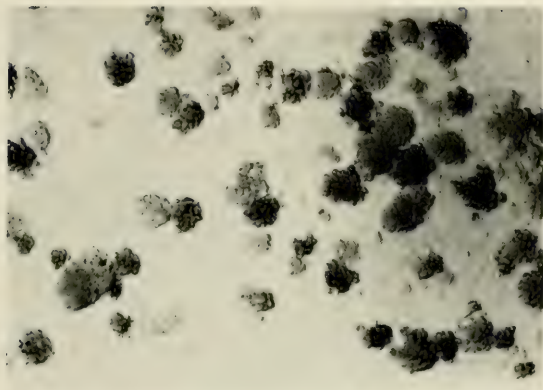


FIGURE 1. Aggregates showing partial covering of *Microciona prolifera* by *Halichondria bowerbanki* aggregates in a suspension of mechanically dissociated cells. The darker aggregates are *Microciona*.

aggregate where one or the other species was concentrated. A mixture of *M* and *B* cells with the *B* factor gave aggregation in only one out of nine trials, and this aggregate contained both *M* and *B* cells. Mixture of both cell types and both factors also resulted in the formation of common aggregates. A suspension of cells from both species failed to aggregate in the absence of either factor. These results are detailed in Table I.

When cells of *Halichondria panacea* (*P*) are substituted for those of *Halichondria bowerbanki*, the results of the above experiments are far more erratic. Cross-aggregation of *P* by *M* factor occurred less often (three out of six trials), and the cells often appeared damaged. The combination of *M* and *P* cells with the *M* factor gave erratic results: sometimes the species formed intermingled aggregates and sometimes separate aggregates formed. It is probable that the factor isolation method used is not optimal for *Halichondria* with respect to both viability and potency of the factor obtained.

Moscona (1968) established that the factor's enhancement of aggregation and its species-specificity in *Microciona* and *Haliclona* persisted even after the fixation of the cells in formalin. The similarity of aggregation of fixed and living cells was also observed here. CMF-dissociated cells were fixed for four hours in 4% glutaraldehyde, washed in MBL-SW and combined as detailed above. The aggregates were much looser than those of living cells and less rounded, but the cells retained the same specific reactivity to factors as living cells.

Mechanically dissociated cells

Gyrationally aggregated mixtures of *M* and *B* cells, at either 4° C or room temperature, formed tight aggregates which, at first glance, appeared to be mono-

specific. Upon closer examination, it was observed that small *M* aggregates were often enclosed within *B* aggregates and that many of the *M* aggregates had several *B* aggregates or a partial halo of *B* cells clustered around them. The converse configuration was never observed. This type of aggregation was particularly obvious in petri dish clusters of an *M* and *B* mixture (Fig. 1). It thus appears that the equilibrium configuration adopted by a mixture of mechanically dissociated *B* and *M* cells is one in which *M* is partially covered by *B*. In gyrationally aggregated cultures this covering may be obscured by shearing forces, which may separate the *M* and *B* aggregates.

Addition of the *M* factor to mechanically dissociated *M* cells resulted in faster and more extensive aggregation, as first reported by Moscona (1968). The *M* factor also enhanced the aggregation of mechanically dissociated *B* cells. The combination of mechanically dissociated *M* cells plus CMF-dissociated stained *M* cells gave intermingled aggregates at both 4° C and room temperature. The aggregates were of the same size as those formed by mechanically dissociated cells in the absence of the added factor. If the factor was added to this combination the aggregates were much larger.

Cell type effects

In CMF-dissociated *Microciconia* suspensions which had been stained with NBS, cells of several kinds could be distinguished. Prominent among these were cells of three types. Certain of the larger cells (called NBS⁺ here) stained an intense blue. These NBS⁺ cells are identical with the "grey cells" of Wilson and Penney (1930) and, when unstained, contain large numbers of small grey vacuoles. The unstained cells in the suspension (termed NBS⁻) consist of two

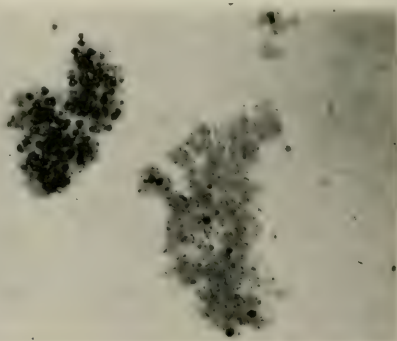


FIGURE 2. Mini-aggregates formed in a gyrated suspension of stained CMF-dissociated *Microciconia* cells. Aggregate at right is typical of the mini-aggregates. The aggregate at the left has a proportion of stained cells similar to that in the large normal aggregates formed in the same suspension. The aggregates have been squashed for photography and are naturally more rounded and compact.

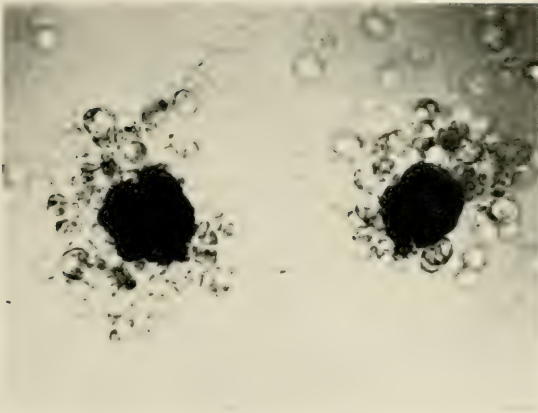
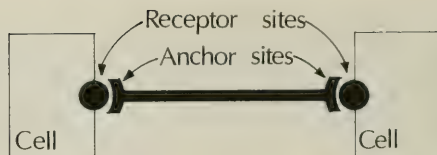


FIGURE 3. Aggregates showing localization of different cell types formed in petri dish cultures of NBS stained, CMF-dissociated *Microciona*. NBS stained "grey" cells cluster in the interior of the aggregates.

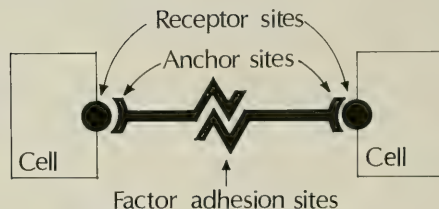
major cell types: orange-vacuolated, nucleolated cells and somewhat smaller, clear cells containing red granules which Wilson and Penney identified as collar cells.

In gyrational aggregates some cells remain unaggregated, some cells form mini-aggregates, and the majority of the cells are incorporated into large aggregates over 40 times the diameter of the mini-aggregates. The unaggregated cells and the cells composing almost all of the mini-aggregates were predominantly (95%) NBS⁻ cells, although a few of the mini-aggregates were composed largely of NBS⁺ cells (Fig. 2). Clumps the size of the mini-aggregates did not persist through the dissociation procedure and must have formed as the result of adhesion of specific cell populations. The large aggregates contained both NBS⁺ and NBS⁻ cells but these types were positioned non-randomly. The NBS⁺ cells formed tight knots or clusters, surrounded by NBS⁻ cells, and were almost always located internally rather than on the surfaces of the aggregates.

In petri dish cultures of *M* cells plus *M* factor similar results were obtained. The NBS⁺ cells again formed tight knots in the interior of the aggregates, surrounded by NBS⁻ cells (Fig. 3). When stain was added to cultures already aggregated, the stain became localized in the same cells as described above. Moreover, the "grey" cells which absorb the stain can be identified upon close examination in unstained aggregates and they are localized similarly. Thus, the differences in aggregating behavior cannot be attributed to the staining procedure which assists in discriminating the cell types. It does appear, however, that there is a difference in behavior of CMF-dissociated and mechanically dissociated cells in regard to the localization of the NBS⁺ cells within aggregates. Wilson and Penney (1930) found, and this was supported by the present study, that some NBS⁺ grey cells



SYMMETRICAL FACTOR



ASYMMETRICAL FACTOR

FIGURE 4. Symmetrical and asymmetrical factors.

form a tight layer on the surface of aggregates of mechanically dissociated cells. Thus the CMF-dissociation procedure apparently alters the localization of cell types.

Using a velocity sedimentation technique (Leith and Steinberg, 1972) it was possible to obtain a very distinct separation of the three cell types. The bottom fraction removed from the chamber consisted primarily of NBS⁺ cells. The central fraction contained the NBS⁻ vacuolated cells and the upper fraction contained the NBS⁻ collar cells. All three types of cells appeared healthy, and aggregated when factor was added. Cells from the NBS⁺ fraction formed compact, rounded aggregates with little enclosed space. Vacuolated NBS⁻ cells formed looser, irregularly shaped aggregates, while the NBS⁻ collar cells aggregated poorly. When the NBS⁻ cell fractions were combined with the NBS⁺ cells in the presence of factor, the aggregates consisted of clumps of NBS⁺ cells surrounded by NBS⁻ cells.

DISCUSSION

The results reported here are consistent with the adhesive role for factor proposed by Humphreys, and they also give insight into a factor's mechanism of action.

A plausible mechanism must explain these observations as simply as possible and also be consistent with the results of earlier workers. As mentioned before, the cell surface must contain receptor sites to which the factor can bind. Indeed, Weinbaum and Burger (1971) have isolated these sites using a hypotonic shock. Moscona (1968) observed, and it is confirmed here, that addition of factor to mechanically dissociated cells results in faster and more extensive aggregation than would otherwise occur. The simplest explanation is that mechanically dissociated cells normally display an excess of receptor sites to which extra factor can crosslink. There is evidence in some species, that failure to aggregate in heterospecific factor is associated with failure of the factor to bind to the receptor sites. Moscona (1968) demonstrated that *Haliclona* cells, which do not aggregate in *Microciona* factor, also do not remove this factor from solution. This indicates that the specificity of the factor-receptor site bond is the determinant of the specificity of the factor's adhesive-promoting ability in these species.

The factor bridge which links the cells' receptor sites could consist of either symmetrical or asymmetrical factor units. That is, a bridge could consist of a factor unit having two (or more) identical anchor sites which recognize complementary receptor sites on cell surfaces to give a symmetrical factor. Or, alternatively, a bridge might consist of two asymmetrical factor units, each of which has both anchor sites for binding to cells and adhesion sites for attachment to a second factor unit (Fig. 4).

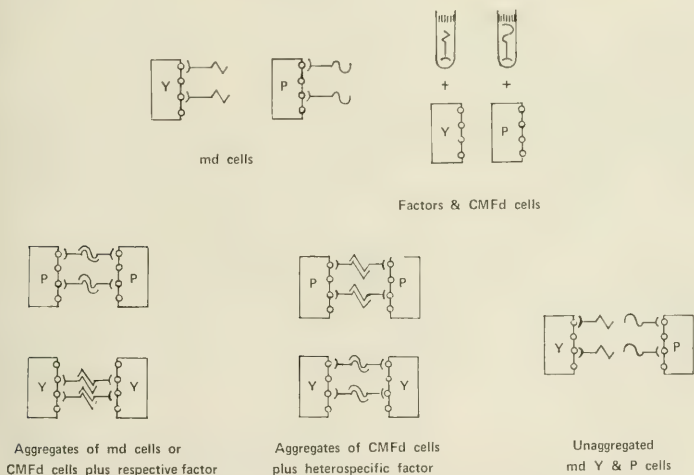


FIGURE 5. An asymmetrical factor mechanism to explain MacLennan and Dodd's observations. The symbol Y denotes *Hymeniacidon perleve* cells, P denotes *Halichondria panicea*, md denotes mechanically dissociated cells and CMFd denotes cells dissociated in calcium-and-magnesium-free sea water.

Previous work can be interpreted in support of the existence of both asymmetrical and symmetrical factors. The results of MacLennan and Dodd (1967) point to an asymmetrical factor. They report a case in which the behavior of the cells and factors resembles that reported here for *Microciona* and *Halichondria bowserbanki*, with one important exception. When mechanically dissociated cells of specimens of *Hymeniacidon perleze* and *Halichondria panicea* were mixed, completely separate species-specific aggregates were formed. Yet the *Hymeniacidon* aggregation factor aggregated CMF-dissociated *Halichondria*. If the factor is symmetrical, it is difficult to explain why mechanically dissociated cells aggregate separately, although their factors promote the aggregation of both species' cells. MacLennan and Dodd's description, if wholly accurate, suggests that the cells have similar receptor and anchor sites but that their factor's adhesion sites are incapable of adhering to the factor of another species (Fig. 5). Since mechanically dissociated cells have only their own factor units on their surfaces, they will be incapable of adhering to cells of the other species. Both factors can bind to the receptor sites of either species, but once bound they only adhere to other factor units of the same species. This is the simplest sort of mechanism which is consistent with the results of MacLennan and Dodd.

The aggregational specificities reported in this paper, as well as those described by Humphreys (1963, 1970), Moscona (1968) and Sara *et al.* (1966) can be accounted for by either an asymmetrical mechanism such as that described above, or by a simpler mechanism using a symmetrical factor. In such a mechanism, the *Microciona* factor is able to anchor to *Halichondria bowserbanki* receptor sites. *Halichondria* factor was difficult to isolate in an active state, but the results indicate that this factor will anchor to *Microciona* cells and cause aggregation. The difficulty with a symmetrical factor mechanism lies in explaining why mechanically dissociated cells of the two species can segregate when mixed, if their factors can adhere to the other's cells. Our observations indicate that *Microciona* and *Halichondria* cells do not segregate completely, but rather form aggregates with a very incomplete degree of partial covering. Steinberg (1964) has shown that joint aggregates of this type would occur if the *Microciona* cells were slightly more cohesive than the *Halichondria* cells, which in turn adhere more strongly to each other than to cells of the opposite species. Mechanistically, this might be accomplished if the adhesion of *Microciona* factor to *Halichondria* receptors is of lower energy than the adhesion to its own receptors, etc. Since the factor on mechanically dissociated cells is able to adhere to cells of either species, there may be a degree of species intermingling initially, however, these cells will sort out later to maximize the total adhesion. A diagrammatic representation of this mechanism is given in Figure 6.

In the above discussion a distinction has emerged between the explanation of the specificity of a factor's action and the explanation of the specificity of aggregation itself. If the factors are symmetrical, factor specificity will of necessity coincide with the aggregational specificity of mechanically dissociated cells, as observed by Humphreys (1970). But if factor is asymmetrical, factor and aggregational specificity need not coincide.

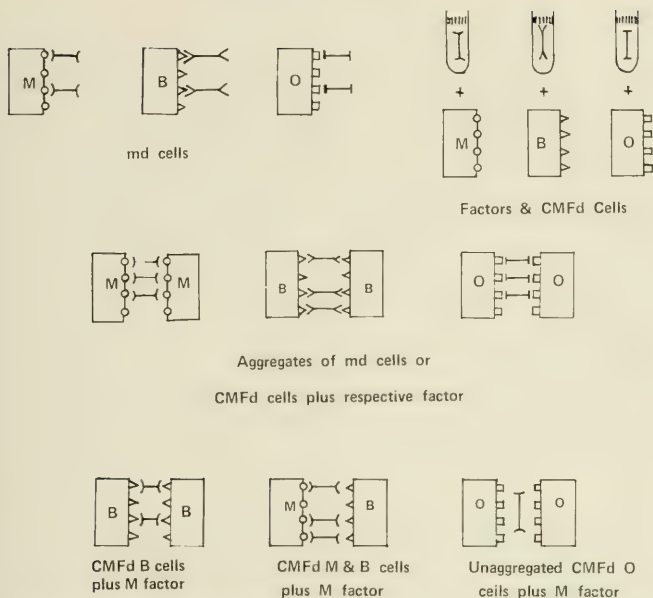


FIGURE 6. A symmetrical factor mechanism sufficient to explain the results reported here. The symbols are the same as in Figure 5, with the addition that B denotes *Halichondria bowyerbanki* and O denotes *Haliclona oculata*. M and B factors adhere more strongly to their own receptors than to each other's.

The observations of differences in aggregational specificity indicate that there are at least three different populations of cells, as distinguished by their aggregating abilities, in dissociated *Microciona* suspensions. The first population is composed of cells which are apparently non-adhesive, as they remain isolated and do not participate in aggregation. They are almost exclusively small NBS⁻ collar cells. They may be a product of injury during dissociation, perhaps with a loss of receptor sites, but death alone—as in fixation—is not sufficient to cause a loss of aggregation ability. If their behavior is due to injury, it must be correlated with an increased sensitivity of the collar cells to dissociation, as even in mechanically dissociated suspensions these cells aggregate poorly.

A second cell population is composed of the large NBS⁺ cells. If the sponge is dissociated using the CMF procedure, these cells localize internally in aggregates. This would be predicted by Steinberg's differential adhesion hypothesis if the NBS⁺ cells were more cohesive than the others. The tight knots which these cells form may be indicative of this cohesiveness. The cohesiveness may be due to an ability

to form a larger number of intercellular linkages rather than an increase in the strength of a single linkage.

The third cell population is composed of the relatively less adhesive NBS⁻ cells. These cells are present in both normal aggregates and in the mini-aggregates formed by CMF-dissociated cells. Separation experiments show that factor promotes their aggregation. The non-random incorporation of NBS⁺ and NBS⁻ cells into the mini-aggregates may be explainable if, in addition to having less cohesiveness than the NBS⁺ cells, the NBS⁻ cells aggregate more slowly. Curtis (1970) proposed that unaggregated cells in a suspension are more likely to adhere to other cells than to a large aggregate due to rheological effects. However, further work may show that the NBS⁻ cells in the mini-aggregates differ in their adhesion from the NBS⁻ cells in the large aggregates and thus comprise a fourth cell population. To explain their differing aggregation it might then be necessary to introduce a more complex factor mechanism having two or more varieties of factor in a single species.

The present results show that adhesion in *Microciona* is more complicated than in a mixture of cells which are uniformly reactive to a factor. There are distinct differences in aggregation ability among cell populations which affect the type and distribution of cells incorporated into aggregates. At present it is not clear whether simple mechanisms of adhesive factor action, such as those discussed earlier, are sufficient to explain all of these differences.

Many of the ideas present here arose from conversations with Dr. Malcolm S. Steinberg and I gratefully acknowledge this contribution. Supported in part by research grant CA-13605 awarded to Dr. Malcolm S. Steinberg by the National Cancer Institute, DHEW.

SUMMARY

A pair of sponge species, *Microciona prolifera* and *Halichondria bozzerbanki*, which lack mutual species specificity in their aggregation "factors", are useful in establishing the mechanisms of action of these factors. These sponges were dissociated both mechanically, which leaves the factor on the cell surface, and by Humphrey's (1963) method, which isolates the factor from the cells. The adhesive specificities which arose, in the various combinations tested, point to an intercellular factor bridge consisting of a single symmetrical unit. An analysis of most other workers' results is consistent with this interpretation. However, MacLennan and Dodd's (1967) results using other species would require a bridge consisting of two or more asymmetrical units.

Differences were found in the specificity of adhesion of various types of cells within a single species. This presents a heretofore unconsidered problem in assessing the adhesive factor's mechanism of action. Three structurally distinct cell types were separated from a suspension of dissociated *Microciona* cells by velocity sedimentation. These cells differ greatly in adhesiveness. The differences in adhesion are correlated with numbers and positions of cells incorporated into aggregates. Such differences are considered in explaining the mechanism of action of the factors.

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SECRETION OF TRYPSIN IN THE OESOPHAGUS OF *NEREIS VIRENS* SARS (POLYCHAETA: ERRANTIA). A BIOCHEMICAL AND HISTOLOGICAL STUDY

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Nereis virens is an errant polychaete which lives in sand galleries that it rarely leaves. Most of the authors studying its feeding behavior consider this annelid to be carnivorous or almost omnivorous (Copeland and Wieman, 1924; Case, 1962; Goerke, 1971a, b; Kay, 1972; Tenore and Gopalan, 1974). Several of these authors succeeded in feeding the annelid with pieces of mollusc or polychaete flesh which are offered at the opening of the gallery. The worm grips the food with its powerful paired jaws and takes it into its burrow before ingestion. This carnivorous mode of feeding suggests that the digestive tract of the worm must be equipped with a complement of proteolytic enzymes for digestion of the flesh food bolus.

A trypsin-like enzyme has been extracted and purified from the digestive fluid of *Nereis brandti* (DeVillez and Reid, 1971) and characterized as a homogeneous component. In a study of distribution of digestive enzymes in the digestive tract of *Nereis virens* (Kay, 1974), the oesophageal pouches (caeca) and the anterior region of the intestine were proposed as main sites of production of proteolytic enzymes. In histochemical tests, most endo- and exo-peptidase activities were associated with the pouches and anterior intestinal regions, whereas the oesophagus showed very weak activity. The present study includes a re-examination for sites of production of trypsin along the digestive tract using biochemical methods, and the investigation of the histological and cytological structure of the secretory area.

MATERIALS AND METHODS

Specimens of *Nereis virens* were obtained from Northeast Marine Specimens Co., Inc., Woods Hole, and maintained in an artificial salt water aquarium at 13° C.

For biochemical studies, starved animals were maintained one week prior to the preparation of homogenates. Pharynx, oesophagus with caeca, and intestine were isolated and rinsed in filtered sea water before extract preparation of 10% homogenates of each organ in distilled water.

For histological studies, animals were maintained at least three days prior to feeding. Animals were force-fed with a disposable pipet a total of 1 ml of a 10% homogenate of commercial fish food pellets. Animals were sacrificed at 1, 12, 24 or 48 hr after the onset of feeding.

The tryptic assay was a modification of the method of Smith and Van Frank (1975) and included the following reagents: 0.6 mg/ml of N-Carbobenzoyxy-diglycyl-arginyl-4-methoxy- β -naphthylamide (Z-Digly-Arg-NNAP-OME)

TABLE I

Tryptic activity in 10% homogenates of the polychaete Nereis virens.

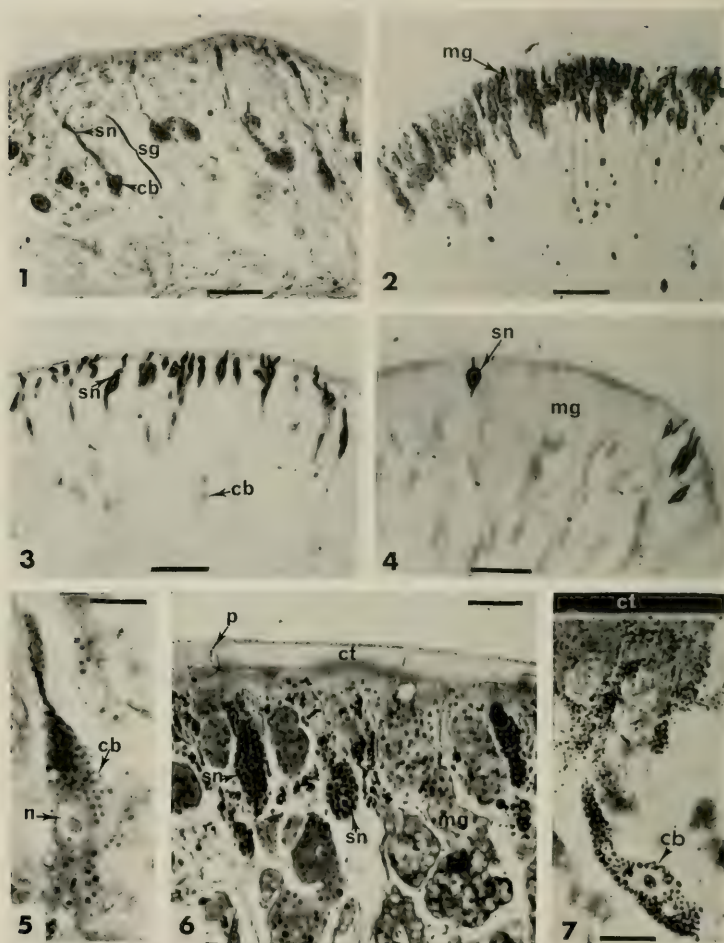
Substrates	Extract No. and treatment	Activity — A_{530} /ml/hr		
		Pharynx	Oesophagus with caeca	Anterior intestine
Z-Digly-Arg-NNAP-OME	No. 1	0.9	13.6	4.1
	TLCK	0.0	0.0	0.0
	PSMF	0.0	0.0	0.0
	No. 2	0.6	10.1	2.2
	TLCK	0.0	0.0	0.0
	PSMF	0.0	0.0	0.0
BZ-Arg-NNAP	No. 1	0.9	5.7	1.1
	TLCK	0.0	0.0	0.0
	PSMF	0.0	0.0	0.0

Bachem Inc., Marina Del Rey, California or 0.4 mg/ml of α -N-Benzoyl-DL-arginine- β -naphthylamide HCl (BZ-ARG-NNAP) Sigma Chemical Co., St. Louis, Missouri, 0.1 mg/ml of O-dianisidine, tetrazotized (Fast blue B), Sigma, and N-2-hydroxyethyl piperazine-N-2-ethane-sulfonic acid (HEPES), Sigma. Tryptic inhibitors included 0.17 mg/ml of phenylmethylsulfonylfluoride, (PMSF), Sigma or 0.74 mg/ml of N- α -p-tosyl-L-lysine-chloromethyl ketone HCl (TLCK), Sigma.

Incubation mixtures consisted of 0.2 ml of 10% homogenate, 0.1 ml of 1% Triton-X-100 and 0.1 ml distilled water or inhibitor. Mixtures were allowed to stand for 30 min at 22 °C. After the addition of 1 ml of substrate buffered with 0.1 M HEPES at pH 8.2, the reaction proceeded for one hour at 37 °C and was stopped with the addition of 0.1 ml of 1 N HCl in 1% Triton-X-100. Absorbance was read at 530 nm 10 min after the addition of 1 ml of a fresh solution of Fast blue B buffered at pH 5.3 with 0.1 M HEPES. The product-dye complex was confirmed by chelation with 2% CuSO_4 , which resulted in a stabilized purple product.

The histological and histochemical studies were performed on isolated parts of the digestive tract fixed in a neutralized 10% formaldehyde-saline solution for six hours, then dehydrated and embedded in paraffin wax. The techniques are summarized in Table II. Most of the histochemical tests for proteins were applied according to the treatise of Ganter and Jolles (1970) and indicated in Table II. Histochemical characterization of mucopolysaccharids was not the aim of this work, the coupled diamine-alcian blue staining of Spicer (1965) was only used to visualize the mucous glands. Reactions were judged negative (—) to strongly positive (+++).

For the cytological observations the tissues were fixed 1½ hr in ice-cold 3% glutaraldehyde in 0.2 M phosphate buffer at pH 7.4 and containing 0.5% NaCl. Samples were rinsed in the salt-buffer solution containing 5.8% glucose and post-fixed for 2 hr in 1% osmic acid in 0.2 M collidine at pH 7.4. Dehydration was performed through acetone before embedding either in a low viscosity embedding medium (Spurr, 1969) or in a mixture of epon-araldite (Epon 812—31 g, Aral-



FIGURES 1-7. Bright Field microscopy of the serous gland cells of the anterior oesophagus in *Nereis virens*.

FIGURE 1. Oesophageal serous gland in a fasting *Nereis virens* (Tetrazoreaction of Danielli). The scale represents 40 μ m.

FIGURE 2. Oesophageal mucous glands stained with Alcian blue in the coupled diamine reaction of Spicer. The scale represents 45 μ m.

dite 502—22 g, DDSA 60 g, DER 732—8.1 g, and DMP 30—4% v v). The semi-thin sections (ca. 1 μm) were stained without removal of the embedding medium, in toluidine blue (Trump, Smuckler and Benditt, 1961), or with the tri-basic technique (Grimley, Albrecht and Michelitch, 1965), or after dissolution of the epoxy medium in sodium alcoholate (Berkowitz, Fiorello, Kruger and Maxwell, 1968), stained with the basic fuchsin-methyl blue (Michel, Mira and Mitrovic, 1973). The ultra-thin sections were counterstained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). Ultrastructural observations were made with a Phillips 301 electron microscope.

OBSERVATIONS AND RESULTS

The distribution of tryptic activity in homogenates is summarized in Table I. Greatest activity was associated with oesophageal-caecal extracts and was inhibited by PMSF and TLCK. It should be noted that only uncentrifuged homogenates demonstrated tryptic activity and the diglycyl-arginyl naphthylamide derivative was a better chromogenic substrate than the arginyl derivative. Because of the high tryptic activity, histological, histochemical and cytological studies of the oesophagus and neighboring areas were initiated.

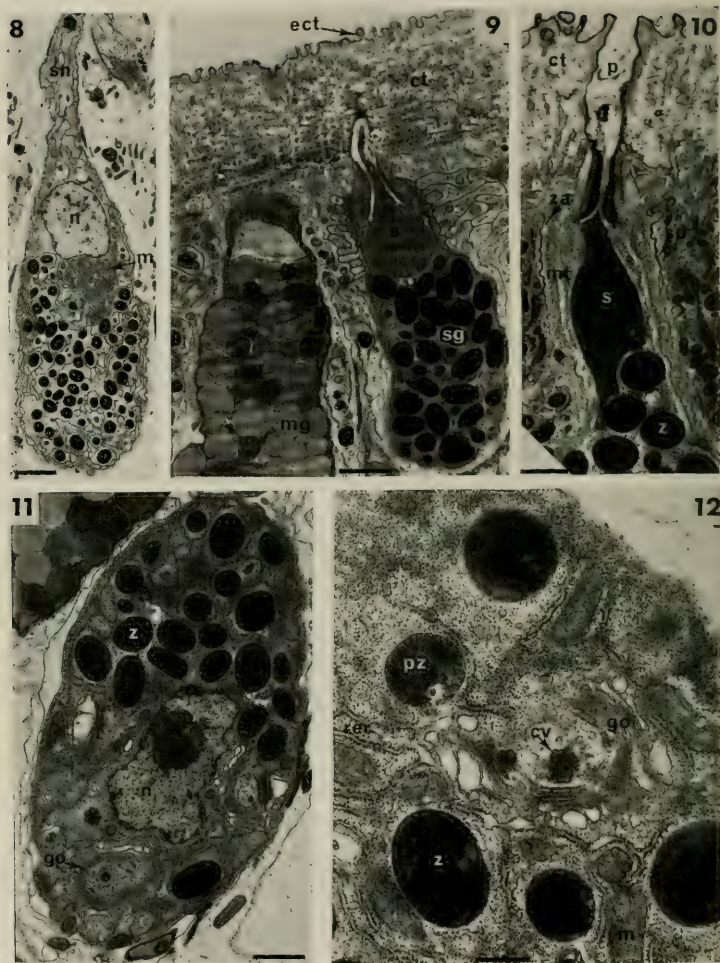
The histological study with azan or haematoxylin stains revealed the presence of a cuticular lining throughout the whole foregut including pharynx, oesophagus, and caeca. Serous gland cells (Fig. 1) are present in the oesophageal wall extending from the short precaecal area past the caeca (including the limited proximal part of the caeca) to a short postcaecal area. Mucous glands exist in the same regions (Fig. 2) and are numerous in the entire caeca. Mucous glands predominate in the posterior oesophagus, where the serous glands are absent. The serous cells (Fig. 1) are scattered in the conjunctive tissue under the epithelium. They are composed of a cell body which contains the nucleus and a long secretory neck which runs between the mucous glands up to the thick cuticle (3.5 μm), where it opens by means of a small pore.

The histochemical study of the serous secretion (Table II), was undertaken to verify whether the serous cells show characteristics comparable to those of the proteinase secreting cells in the pharynx of the polychaete *Eulalia viridis* and the mammalian exocrine pancreas. The results are summarized in Table II and demonstrate the presence of large amounts of tryptophan and cystine amino acids in the serous secretion, as in the previously mentioned organs. A secretory cycle parallel to the digestive cycle has been shown in these cells. In a fasting animal (Fig. 1), the whole cell is filled with secretory granules; 1 hr after a meal (Fig. 3) the cell body is empty and the secretion is present only in the secretory neck; 12 hr after

FIGURES 3 AND 4. Serous glands stained according to Glenner's rosindole reaction: 1 hr after meal (3), 12 hr after meal (4). The scales represent, respectively, 50 μm (3) and 40 μm (4).

FIGURE 5. Body of a serous cell, 24 hr after meal, stained according to Klessen's reaction for zymogen granules. The scale represents 7 μm .

FIGURES 6 AND 7. Semi-thin sections in the anterior oesophagus of a fasting *Nereis virens* stained with toluidine blue (6) and with basic-fuchsin methyl blue (7). The scales represent 10 μm for both figures. Abbreviations are: cb, cellular body; ct, cuticle; mg, mucous gland; p, pore; sg, serous gland; sn, secretory neck.



FIGURES 8-12. Electron micrographs of the gland cells in the anterior oesophagus of *Nereis virens*.

FIGURE 8. Body of a serous gland with the beginning the secretory neck. The scale represents 4 μ m.

TABLE II

Histological stainings and histochemical reactions in the oesophageal gland cells of Nereis virens.

Staining Techniques	Reference	Results		Indicated
		Serous glands	Mucous glands	
Histological stainings				
Azan	Heidenhain	Red +++	Blue +	Serous—red, Mucous—blue
Haematoxylin-trichromic	Prenant	Black +++	Green ++	Serous—iron haematoxylin
Semi-thin sections				
Toluidine blue	Trump <i>et al.</i> (1961)	Blue +++	Violet +	Serous—blue, Mucous—(Metachromatic)
Tribasic staining	Grimley <i>et al.</i> (1965)	Green +++	Red +	Serous—green
Basic fuchsin, methyl blue	Michel <i>et al.</i> (1973)	Red +++	Blue +	Serous—red
Histochemical reactions				
Ninhydrin-Schiff	Yasuma-Ichikawa (1953)	++	—	Proteins (NH ₂)
Dinitrofluorobenzene	Danielli, var. Burstone (1955)	++	—	NH ₂ , SH, phenol and imidazol
Tetrazoreaction	Danielli (1947)	+++	—	Aromatic and SH
Rosindole reaction	Glennier (1957)	+++	—	Tryptophan
Alkaline tetrazolium	Pearse (1953)	+++	—	SH and S-S
Oxidized diamines	Klessen (1972)	+++	—	(zymogen granules), S-S (cystine)
D.D.D.	Barnnett-Seligman (1954)	+	—	SH (cysteine)
R.S.R.	Bennet (1951)	+	—	SH (cysteine)
Thioglycolate-D.D.D.		+++	—	SH and S-S
Thioglycolate-R.S.R.		+++	—	SH and S-S
Diamines-Alcian blue pH 2.5	Spicer (1965)	—	Blue ++	Carboxylated mucus—blue
Conclusions		Protein secretion tryptophan cystine	Mucus secretion	

a meal most of the serous gland cells are empty and a few secretory necks contain secretion in their apices (Fig. 4); 24 hr after a meal the cell body actively secretes new serous granules (Fig. 5). Based on these observations, it is proposed that the serous granules are zymogen granules.

The observations on semi-thin sections stained with toluidine blue (Fig. 6) or with the basic fuchsin-methyl blue (Fig. 7) provide a clear distinction between the serous and mucous cells in the material fixed for electron microscopy. The serous cells contain one type of oblong zymogen granule which stains blue with toluidine blue and bright red with the basic fuchsin, which is known to stain the protein secretion in the pharynx of *Eulalia viridis* (Michel, 1972). The mucous glands have granules of various sizes and opacities which are stained metachromatically in violet with toluidine blue and take faintly the methyl blue in the dichromic staining.

FIGURE 9. Apical parts of the mucous and serous glands. The scale represents 1.7 μ m.

FIGURE 10. Details of the neck and the pore of a serous gland. The scale represents 1.2 μ m.

FIGURE 11. Body of a serous gland cell. The scale represents 1.1 μ m.

FIGURE 12. Secretory area in the body of a serous gland. The scale represents 0.5 μ m.
Abbreviations are: cv, condensing vacuole; ct, cuticle; ect, epicuticle; go, golgi apparatus; m, mitochondria; mg, mucous granules; mt, microtubules; n, nucleus; pz, prozymogen granules; rer, rough surfaced endoplasmic reticulum; s, secretion; sd, septate desmosome; sg, serous gland; sn, secretory neck; z, zymogen granules; and za, *zonula adhaerens*.

The ultrastructure of the serous cells is provided in Figures 8 through 12. The body of a cell contains a large nucleus with prominent nucleolus and peripheral chromatin (Fig. 11). Adjacent to the nucleus are seen mitochondria (m) (Fig. 8), rough-surfaced endoplasmic reticulum (rer) and Golgi apparatus (go) (Figs. 11, 12). Mature zymogen granules (z) and free ribosomal granules are scattered between reticular cisternae.

It seems that the zymogen granules appear first as condensing vacuoles (cv) in the mature face of the Golgi's stacks (Fig. 12), but it is also possible that the dilated cisternae of the rough endoplasmic reticulum take a part in the formation of the granules. The prozymogen granules (pz) have an incomplete membrane and a heterogeneous content. The mature zymogen granules are lined with a unit membrane; they have an oblong shape, the long axis measures up to 1 μ m, and their content is heterogeneous, showing a dense spiral line.

Both serous and mucous glands reach the cuticle (Figs. 9, 10). The anterior oesophageal cuticle shows the same structural aspects as the bodywall cuticle, previously described by several authors (Pilato, 1964; Brökelmann and Fischer, 1966; Storch and Welsch, 1972); successive perpendicular sheets of collagen fibers are crossed by epithelial microvilli which reach the epicuticle (ect). The surface of the epicuticle has indentations lined by a denser external sheet. Below the cuticle, the apex of the secretory neck possesses two lateral *zonulae adhaerentes* (Za) and lateral septate desmosomes (sp) which are frequently convoluted. The apex of the gland cells does not possess microvilli as in the epidermal glands, but the pore is also lined by "an intucking of the epicuticle" (Dorsett and Hyde, 1970). The deepest part of the pore shows a thickening of its wall when entering the apex of the gland cell (Fig. 10). Microtubules (mt) are present peripherally near the septate desmosomes. The secretion is not emitted in the form of zymogen granules; at the apex of the neck the membrane of the granules bursts and the secretion appears as a dense, confluent mass which can be easily emitted through the cuticular pore into the oesophageal lumen.

DISCUSSION

The present results implicate the oesophagus as the main site of production of the tryptic digestive enzyme and are consistent with the quantitative estimation for general proteolytic activity reported by Kay (1974); previously reported high endo- and exo-peptidase activities in the oesophageal pouches (caeca) and anterior intestine may have been due to intracellular proteinases with functions other than extracellular digestion. It must be noted that caecal homogenates alone were not assayed for trypsin in the present study. However, the high tryptic activity of the oesophagus correlates with the distribution of serous glandular cells in the anterior oesophagus near the caecal junctions and in the limited part of the proximal caeca.

The presence of tryptic activities in the digestive tracts of polychaetes has been reported for several species as recently reviewed (Michel and DeVillez, 1978), but reports about the foregut (ectodermal origin) are less frequent. The presence of serous cells secreting a tryptic-like proteinase has been shown along the entire

pharynx of the phyllodocid *Eulalia viridis* (Michel, 1968, 1969, 1972). The histochemical characteristics of oesophageal serous glands of *N. virens* are the same as those of the pharynx of *E. viridis* or the exocrine gland cells of the mammalian pancreas (Pearse, 1960). However, several differences must be noted in the cytological aspects. First, in the pharynx of *E. viridis* and in the mammalian exocrine pancreas, the zymogen granules have a round form and a dense homogeneous content (see Pfeiffer, Rowden and Weibel, 1974) while the oesophageal zymogen granules of *N. virens* are oblong and show a dense spiral condensation. Secondly, in the pharynx of *E. viridis*, the gland cells are situated just below the cuticle; they possess neither secretory neck nor pore and the secretion must filter across the cuticle by means of the cell microvilli which reach the epicuticle. Third, in the mammalian pancreas, the confluent granules emit the secretion into the acinar lumen by means of the apical microvilli (see Sandborn, 1970). For *N. virens*, the secretion is confluent in the cell apex before it is discharged in the oesophageal lumen.

It is not possible in this communication to substantiate the new concept of "GERL" as recently described by Novikoff, Mori, Quintana, and Yam (1977). However, the formation of the zymogen granules seems to conform to the descriptions given by most of the authors who have studied exocrine pancreas (Case, 1978).

In conclusion, the apparent secretion of trypsin from the oesophagus of the polychaete *N. virens* shows a new example of the presence of a digestive proteolytic enzyme in the foregut of an annelid.

Thanks are due to Miss Rona Long and Miss Maureen Rainey for their contributions to the biochemical and histochemical studies respectively.

SUMMARY

1. The sites of production of a specific digestive enzyme, trypsin, were examined in the digestive tract of the polychaete *Nereis virens*. Additional histochemical and ultrastructural observations were also made.

2. Homogenates of pharynx, oesophagus with caeca and anterior intestine were incubated with N-CBZ-diglycyl-arginyl-4-methoxy- β -naphthylamide at pH 8.2. Reaction product was coupled with Fast Blue B at pH 5.3 and analysed spectrophotometrically at 530 nm. Only extracts of oesophagus with caeca exhibited high tryptic activity, and the reaction was inhibited in the presence of phenylmethylsulfonylfluoride and N- α -p-tosyl-L-lysine-chloromethyl ketone.

3. Two types of glandular cells were distinguished in the anterior oesophagus and proximal part of the caeca after histological and histochemical stainings. Mucous glands were present. Serous glands demonstrated a granular secretion which stained histochemically like that of vertebrate pancreatic exocrine cells and showed a cyclic activity parallel with the digestive cycle of the annelid.

4. The ultrastructural aspects of the serous glandular cells were examined and compared with the glandular cells of the pharynx of another polychaete, *Eulalia viridis*, and with vertebrate pancreatic exocrine cells.

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THE MORPHOLOGY, LIFE-HISTORY, AND TAXONOMIC
RELATIONS OF *ODHNERIA ODHNERI* TRAVASSOS,
1921 (DIGENEA: MICROPHALLIDAE)¹

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The genus *Odhneria* was erected by Travassos (1921) to receive a new species, designated *Odhneria odhneri*, from the yellow-crowned night-heron, *Nyctanassa violacea*, taken in Manguihos (Rio), Brazil. The genus was included originally in the subfamily Brachycoeliinae Looss, 1899, family Brachycoeliidae Johnston, 1912, which contains parasites of cold-blooded hosts. Yamaguti (1958) noted the agreement between *Odhneria* and members of the subfamily Maritreminae Nicoll, 1909, family Microphallidae Travassos, 1920, which contains chiefly parasites of birds. Accordingly, he transferred *Odhneria* to Maritreminae. This disposition was accepted and *O. odhneri* was redescribed briefly by Travassos, Teixeira de Freitas, and Kohn (1969). Sinclair (1971) next reported *O. odhneri* from 14 species of shorebirds, eight taken in the region of Woods Hole, Massachusetts and six at Sapelo Island, Georgia.

MATERIAL AND METHODS

The discovery of encysted metacercariae in the abdominal muscle of the common prawn, *Palaeomonetes vulgaris*, from Quissett harbor near Woods Hole, initiated the investigation. Incidence of infection in the shrimps was heavy, about 75% of those taken were parasitized, and as many as 90 cysts were found in a single individual. The older, larger specimens were most heavily infected and the young, half-grown ones had few or no cysts. In a shrimp, the cysts were of varying sizes, (Figs. 3, 8), forming a continuous series from minute ones, 0.014 by 0.017 mm, to mature cysts 0.34 mm in diameter. The size differences signified repeated reinfection over a long period of time, and suggested that the snail-host was shedding cercariae during much of the year. Large cysts are enclosed in a weak, fibrous capsule, deposited by the host. The cyst wall is composed of two layers, equal in thickness, each measuring 0.020 to 0.030 mm. The large cysts, (Figs. 4, 9) are clear, translucent, hard, tough, and bounce like marbles. When left for 48 hr in half-tap-half-seawater at 38° C., the wall is weakened and easily ruptured. Under coverglass pressure, the metacercariae escape. The worms (Fig. 5) almost complete their growth in the cyst. Their morphology clearly marks them as microphallid parasites of an avian or mammalian host.

About 50 large cysts were fed to a white mouse and 10 excysted worms were recovered from the rectum 36 hr later. They were active, but free in lumen, in the midst of fecal material, and clearly on the way to elimination. There was no

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development in the mouse and it is probable that many worms had already been discharged. Attempts to obtain mature worms by maintaining metacercariae in natural or dilute seawater at 38° were not successful.

Thirty large cysts, removed from *P. vulgaris*, and some 50 additional ones in living shrimps, were fed to a recently-hatched gull, *Larus argentatus*. The bird was autopsied three days later and 32 gravid worms (Figs. 1, 2), showing only slight differences in size, were recovered from the intestine. As no immature worms were found, it appears that the worms in the small cysts did not survive. Morphology of the mature worms definitely placed them in the genus *Odhneria*, and they are assigned to *O. odhneri*, despite certain misgivings as to the limits of the species.

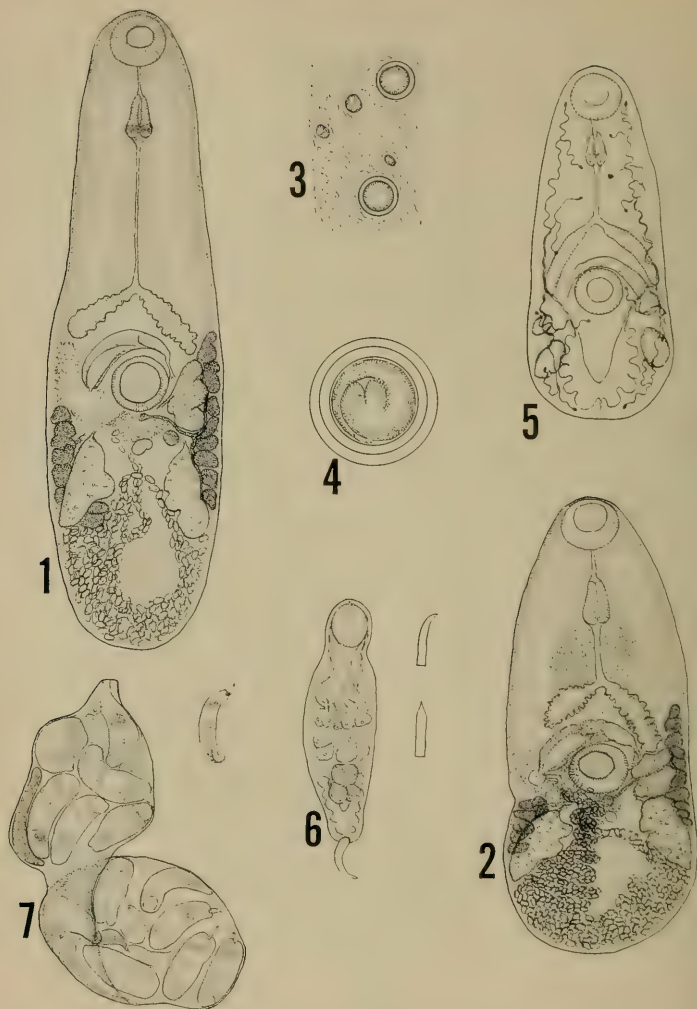
From this point, the investigation was concerned with the source of the encysted metacercariae in *P. vulgaris*. The heavy infection of prawns at Quissett harbor presaged abundance of the molluscan host in the area. Since microphallid cercariae are very small and do not swim for more than 24 hours, the intermediate host must be numerous and shedding large numbers of cercariae. Accordingly, collections of all common gastropod species have been made for two years. Thousands of snails have been taken from areas where the shrimps are numerous and heavily infected. The snails were maintained in fingerbowls and the bowls examined on alternate days for emergent cercariae. If no cercariae, other than well-known species, appeared in three or four weeks, the snails were discarded. At the same time, small and medium sized *P. vulgaris* were kept for possible experimental infection. They were maintained in large jars, provided with food and compressed air and proved very hardy.

Examination of the water in bowls, after the snails had been transferred, did not yield cercariae that could be larvae of *O. odhneri* and eventually the method was abandoned. Crushing and examination of snails, five at a time, a line procedure, provided a rapid and effective means of determining the incidence and identity of infection in each species by larval trematodes. This method proved successful. A crushed *Littorina saxatilis* yielded a liver with sporocysts (Fig. 7) and the sporocysts, when examined with higher magnification, disclosed a cercaria (Fig. 6) with typical microphallid features. The cercariae were very small, transparent, with short tails, and quite unable to swim. They had simple stylets, four pairs of penetration glands, no acetabulum, pharynx or digestive caeca, a V-shaped excretory vesicle, and agreed perfectly with the youngest encysted metacercariae from *P. vulgaris*. Sporocysts and cercariae were studied alive and fixed for stained, permanent preparations. Some of the liver, with sporocysts and cercariae, was fed to a small, half-grown *P. vulgaris* which had been in the laboratory for more than two months. The shrimp had been fed regularly and ate the snail tissue avidly. Dissection of the shrimp three weeks later yielded many recently encysted metacercariae morphologically identical with those of natural infections. It is possible, even probable, that the cercariae from *L. saxatilis*, lying motionless on the bottom of a bowl, may have been overlooked in previous examinations of the snails.

DESCRIPTIONS

Adult (Figs. 1, 2)

The worms, developed for three days in a young *L. argentatus*, are not fully mature since the uterine coils do not completely fill the space between and behind



the testes. They vary from 0.60 to 1.10 mm in length and 0.24 to 0.32 mm in width. The body is ovate to clavate, wider posteriorly, with rounded ends. The acetabulum is located immediately posterior to the midbody and measures 0.07 to 0.085 mm in diameter. The tegument is heavier anteriorly, measuring as much as 10 μ in thickness at the level of the oral sucker and 2 μ near the posterior end of the body. Near the anterior end of the body there are broad, thin, closely set spines, 6 to 7 μ long, which diminish in size posteriorly and are absent posterior to the testes. The body wall comprises the usual circular, longitudinal and oblique fibers and the parenchyma is relatively loose.

The oral sucker is subterminal and approximately the same size as the acetabulum. In living specimens, the diameter depends on the degree of compression from the cover-glass as evaporation reduces the amount of water in the preparation. In mounted specimens, the size is dependent on the degree of flattening during killing and fixation. The prepharynx is variable in length, usually about one-half the diameter of the oral sucker. The pharynx is oval, 0.055 to 0.060 mm long and 0.035 to 0.045 mm wide; the posterior half contains a large number of nuclei. The esophagus is lined with tegumental membrane and varies in length from 0.10 to 0.20 mm with retraction and extension of the forebody. The digestive caeca are lined with tall epithelium, diverge at a wide angle and are preacetabular. In most specimens they are about as long as the esophagus. Contraction of circular muscles in the walls of the caeca may produce outpocketings and a crenate appearance.

The excretory system was worked out in the metacercaria and does not change in the adult condition.

The testes are opposite, oval, 0.10 to 0.145 by 0.06 to 0.09 mm in diameter, located at the lateral margins of the body in the posterior half of the anterior third and all of the middle third of the distance from the level of the acetabulum to the posterior end of the body. They are lobed, longer in the anteroposterior axis, inclined with the anterior ends directed mediad, and in part separated from the body wall by vitelline follicles. Sperm ducts arise from the anterior ends of the testes and converge to join in the interval between the median face of the ovary, the right edge of the acetabulum and the posterior end of the right caecum. The common duct enters the right end of the cirrus sac, a muscular organ 0.15 to 0.20 mm long and 0.03 to 0.04 mm in diameter, which arches across the body anterior to the acetabulum, and opens into the genital atrium located between the acetabulum and the sinistral body wall. Within the cirrus sac, the male duct expands to form an oval seminal vesicle, 0.06 to 0.08 mm long and 0.02 to 0.025 mm wide, which is followed by a short, sometimes curved or sinuous duct that opens

FIGURE 1. Mature specimen, dorsal view, extended, 1.10 mm long.

FIGURE 2. Mature specimen, dorsal view, retracted, 0.78 mm long.

FIGURE 3. Abdominal musculature of *P. vulgaris* with five metacercariae, natural infection. The largest is 0.30 mm in diameter; the smallest, 0.07 by 0.06 mm.

FIGURE 4. Metacercaria in double walled cyst; 0.34 mm in diameter, wall 0.06 mm thick, cavity, 0.22 mm in diameter.

FIGURE 5. Metacercaria, excysted, ventral view, 0.07 mm long, excretory system added from study of living specimens.

FIGURE 6. Cercaria, from *L. saxatilis*, body, 0.09 mm long. Fixed and stained specimen.

FIGURE 7. Sporocyst from *L. saxatilis*, 0.40 mm. long, 0.16 wide.

into the thick-walled prostatic section of the cirrus sac, 0.06 to 0.09 mm long. The lumen of this section is expanded and ends in a short duct that leads to the genital atrium which measures 0.03 to 0.05 mm in diameter and is enclosed in secretory cells.

The ovary is located on the right side at the level of or slightly posterior to the acetabulum. It is oval, longer in the anteroposterior axis, to almost spherical, 0.065 to 0.10 mm in diameter, lobed, near or contiguous to the right testes. The oviduct arises from the median face of the ovary and passes mediad along the posterior face of the acetabulum. Near the midline it receives a duct from the seminal receptacle, almost immediately another from the vitelline receptacle and then enters Mehlis' gland. From the seminal receptacle, Laurer's canal leads to the dorsal surface of the body. From Mehlis' gland, the uterus passes posteriad and toward the left, winds posteriad along the medial face of the left testis, coils about and passes forward along the medial face of the right testis, then crosses to the left behind Mehlis' gland and becomes the metraterm, the terminal section of the uterus. The metraterm is short, 0.04 to 0.06 mm long and opens into the medial face of the genital atrium. The vitellaria consist of oval to irregular follicles, 0.02 to 0.028 mm in diameter, located on the lateral margins of the body. On the right side they extend from a level anterior to the ovary to near the posterior end of the testis; on the left side from the level of the genital atrium to about one-half the distance from the acetabulum to the posterior end of the body. They are ventral to the testes, which may partially overlap the most posterior follicles. Longitudinal ducts pass along the median faces of the follicles, receive a short duct from each, and at the level of Mehlis' gland, transverse ducts pass mediad and join to form the vitelline receptacle, from which a common duct transmits vitelline cells to the oviduct as it enters Mehlis' gland. Typically, there are ten follicles on the right side and eight on the left side. If the body is extended, the follicles are aligned in a single file; when retracted, the follicles may be disposed in clusters, suggesting a rosette arrangement. Eggs in the initial portion of the uterus have thin, flexible shells and the embryos are readily stained, so the early course of the uterus can be observed; later the shells become thick, hard and opaque. Eggs in the terminal part of the uterus measure 0.017–0.018 by 0.011 to 0.012 mm.

Metacercaria (Figs. 3, 4, 5, 9)

Exposure of a half-grown *P. vulgaris*, that had been in the laboratory for two months resulted in the recovery, after 3 weeks of metacercariae as small as 0.017 by 0.014 mm. In natural infections, cysts of different sizes are usually present, showing continuous, repeated infection. The worms virtually complete their development in the cyst and except for the maturation of the reproductive organs, have attained full size. Examination of excysted specimens permits recognition of the gonads and elucidation of details of the excretory system. The excretory pore is terminal and a short duct, surrounded by a sphincter, leads to the base of the V-shaped vesicle. The wall of the vesicle may have prominent lateral evaginations and the lumen is filled with refractive droplets and cells that have been sluffed from the wall. Released from their cysts, the contents of the vesicle are extruded in a cloud through the excretory pore. From the anterior tips of the vesicle, primary

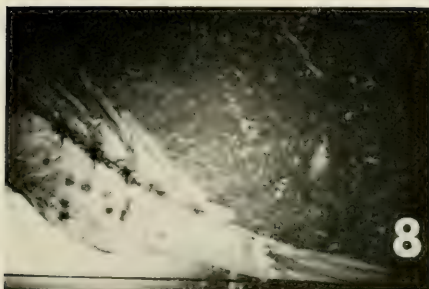


FIGURE 8. Photograph of abdominal musculature, (*Palaeomonetes vulgaris*) with cysts of natural infection.

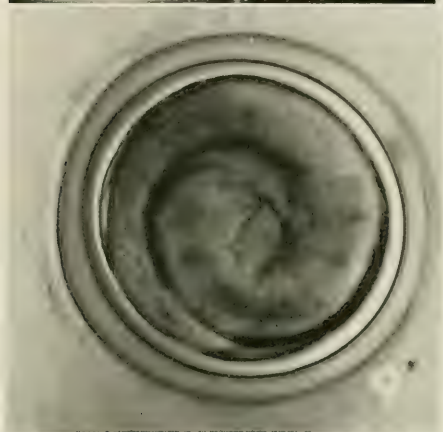


FIGURE 9. Photograph of an encysted metacercaria, 0.34 mm in diameter.

collecting ducts pass anterior and laterad. At the level of the acetabulum they divide into anterior and posterior secondaries. Each secondary gives off a tertiary branch and continues toward the end of the body. Each tertiary branch receives the capillaries from two flame-cells and the formula is $2[(2 + 2) + (2 + 2)]$.

Sporocyst (Fig. 7)

The sporocysts are oval, the largest 0.40 mm in length and 0.16 mm in width. The birth pore is terminal, surrounded by a sphincter and the end bearing the pore may be protruded and somewhat pointed. The wall of the sporocyst is thin, with a single or double layer of nuclei, although cell-boundaries are indistinct or absent.

It contains muscle fibers and the sporocyst may manifest slow, slight movement. Such sporocysts contain as many as 20 well formed cercariae and a few germ balls. Others are somewhat smaller.

Cercaria

Alive, the cercariae are 0.05 to 0.13 mm long and 0.025 to 0.040 mm in width, with a very small tail, 0.015–0.025 mm long and pointed at the tip. Fixed, stained and mounted the average size is 0.08 by 0.034 mm. The oral sucker is 0.025 mm in diameter; the stylet (Fig. 6), embedded in the dorsal wall is narrow, without thickening, 0.015 mm long and 0.002 mm wide, with the tip turned ventrad. The pharynx and digestive caeca are not formed. There are two pairs of penetration glands on each side, located near or slightly posterior to midbody. The cell contents and ducts of the anterior pair stain with neutral red; the posterior cells do not stain. The ducts from the two anterior cells on each side run forward for a short distance and then separate, the duct from the anterior of the two cells turns mediad but the two ducts merge near the oral sucker and pass around the sucker. The ducts from the posterior pair of cells accompany the one from the anterior pair that passes laterally. The ducts from the four cells curve around the sucker, the one from the most anterior cell crosses the other three and opens ventrally, slightly posterior and mediad of the openings of the other three anteriorly beside the stylet. The excretory pore is terminal, closed by a sphincter, and the vesicle is V-shaped, with slight evaginations on the lateral walls. The disposition of the tubules is similar to that of the metacercaria except that the tertiary branches receive the capillary from a single flame-cell and the formula is $2[(1 + 1) + (1 + 1)]$.

DISCUSSION

The first intermediate host of *Odhneria odhneri* in the Woods Hole region is the snail known locally as *Littorina saxatilis*, but its identity is suspect. The species is variant; the snails live high on the rocks, often out of water for days. Some of the specimens are ovoviviparous, with embryos in the brood-chambers, and others appear to be oviparous, but whether or not this is a seasonal phenomenon is doubtful. Probably the species is not identical with *L. saxatilis* (Olivieri, 1792) "among the rocks" at Venice, Italy. Indeed, Heller (1975) predicated that in Wales, *L. saxatilis* is replaced by "four separate, fully sympatric species, *L. rudis*, *L. patula*, *L. nigrolineata* and *L. neglecta*." The species at Woods Hole agrees best with Heller's description of *L. rudis*.

The life-history of a microphallid trematode, identified provisionally as *Levinseniella cruzi* Travassos, 1921, was reported by Young (1938) from southern California. The final hosts were shore-birds, *Limosa fedora* and *Catoprophorus semipalmatus*. The asexual stages were in the snail, *Olivella biplicata*, and the metacercariae in the sand-crab, *Emerita analoga*. Young reported seasonal emergence of cercariae, abundant in late winter and spring but none in fall and early winter, although the snails harbored sporocysts, and cercariae began emerging in January. These findings suggest the crabs acquired the infection in the spring when the cercariae were plentiful; the metacercariae matured during the summer

and the birds became infected during their fall migration. Since cercariae did not emerge until January, the snails must have acquired the infection in the fall.

A similar account of seasonal infection by *Odhneria odhneri* was reported by Sinclair (1971). He recorded infections by *O. odhneri* in 14 species of shore-birds taken at Woods Hole, Massachusetts and Sapelo Island, Georgia. He stated, p. 981, "*Odhneria odhneri* Travassos, 1921, displays seasonality, not in occurrence as such, but of time of infectivity to host. The life-cycle of the organism is unknown. Host records, however, appear to suffice for portraying this feature. Immature specimens have been recovered during mild winters and in spring, the preponderance in late spring. Host species are absent after late spring and most of summer from indicated collecting areas. No immature specimens are found during late summer and fall migrations in either area nor in wintering populations of the Georgia coast. *Odhneria odhneri* displays a continuing growth pattern throughout its adult life which is apparently of 1 year or less in duration. Specimens recovered from some host species in late fall were sluggish, nearly moribund. Residing in the intestinal caeca of the host, these weakened individuals are not flushed out as they would be in the harsher environment of the intestine. In all but the willet, *Catoptrophorus semipalmatus*, intensity and prevalence of infection in hosts decrease as fall progresses."

Through the kindness of Dr. Sinclair, I have examined specimens in his collection taken from *Melanitta deglandi*, *Crocethia alba*, *Catoptrophorus semipalmatus*, *Erolia alpina*, *Ereunetes pusillus*, *Squatarola squatarola*, and *Calidris canutus*. These specimens manifest slight morphological variations but all are clearly specifically identical. The wide range of host species, all migrant birds, is a feature of much ecological significance. The data of Sinclair, that mature specimens of *O. odhneri* are present in birds only during the spring and summer, imply that the snails become infected during the spring migration of the birds when they are shedding eggs of the parasite. Since mature worms have been lost and only immature specimens are present in birds during winter, the infection was acquired during the fall migration. Snails infected in the spring would harbor sporocysts in midsummer and liberate cercariae only in late summer and fall, when *Palacmonetes* become infected. This postulate may explain possible absence of cercariae of *O. odhneri* during midsummer at Woods Hole.

Stunkard (1960) discussed the problems of generic and specific determination of microphallid trematodes. He reviewed the criteria that had been proposed, noted that certain of them are inappropriate, others inadequate, and that the taxonomic difficulties result from incomplete and inadequate data on the morphology, physiology, developmental cycles, and host-parasite relations of these worms. *Odhneria* is a member of the subfamily Maritreminae Nicoll, 1907, family Microphallidae Travassos, 1920. Yamaguti (1971) listed 12 genera in the subfamily, chiefly parasites of birds. But generic limits are often indefinite, uncertain, and frequently overlapping. The genera are distinguished primarily on the location, distribution and arrangement of the vitellaria, which are variable, shifting with contraction and relaxation of different groups of muscles. The validity of certain genera is questionable and *Maritrema* Nicoll, 1907; *Odhneria* Travassos, 1920; *Pseudospelotrema* Yamaguti, 1939 and *Maritreminoides* Rankin, 1939, especially, have presented difficulties.

Pseudosplotrema was based on *P. japonicum* Yamaguti, 1939 from shore-birds in the far east. Yamaguti (1971) included also *P. uriae* Yamaguti, 1939; *P. ammospizae* Hunter and Vernberg, 1953; *P. macrovesicula* Chen, 1957 and its synonym *P. indicum* Bhargwaj, 1962; *P. charadrii* Cable, Connor and Balling, 1960; and *P. nyctanassa* Cable, Connor and Balling, 1960. Rankin (1939) erected the genus *Maritreminoides* with *Maritrema nettac* Gower, 1938 as type-species, and included *Maritrema medium* (Van Cleave and Mueller, 1932) and *Maritrema obstipum* (Van Cleave and Mueller, 1932) as additional species. *Maritrema* Nicoll (1907) was based on *M. gratiosum* Nicoll (1907) and Deblock (1971) listed 35 described species.

The history of *Odhneria* was reviewed by Deblock (1972). He noted that Baer (1943) proposed synonymy of *Maritreminoides* and *Pseudosplotrema*. Etges (1953) proposed synonymy of *Pseudosplotrema* and *Maritrema*. Dery (1958) had described *Maritreminoides raminellae*, a species that was transferred to *Maritrema* by Deblock and Van Ky (1966), to *Pseudosplotrema* by Ching (1963) and by Belopol'skaia (1963) and to *Odhneria* by Yamaguti (1971). *Pseudosplotrema charadrii* Cable, Connor and Balling (1960) was transferred to *Odhneria* by Heard (1970) but Yamaguti (1971) retained the species in *Pseudosplotrema*. *Pseudosplotrema ammospizae* Hunter and Vernberg (1953) was transferred to *Maritreminoides* by Etges (1953) and by Sarkisian (1957), to *Maritrema* by Deblock (1971), but Yamaguti (1971) declared, p. 609, "I believe that the original assignment is correct." After reviewing the history of *Odhneria*, Deblock (1972) presented a reexamination and redescription of the genus and the species included in it. He listed five criteria to distinguish between *Odhneria* and *Pseudosplotrema* but the presumed differences are so tenuous that the distinction is not convincing. When Deblock wrote the text of his (1972) account, he did not have the report of Sinclair (1971) on the finding of *O. odhneri* in 14 species of shore-birds on the Atlantic coast of the United States. Noting variations and overlapping features, Sinclair (1971) predicated synonymy of *O. odhneri*, *Maritreminoides raminellae* Dery, 1958, *Pseudosplotrema charadrii* Cable et al, 1960, and *Odhneria limnodromi* Schell, 1967.

Deblock (1971) recognized the genus *Maritreminoides* with *M. nettac* (Gower, 1938) as type species. However, on the publication of a figure of the type-specimen and a revision of the generic characters by Yamaguti (1971), Deblock (1973) declared, p. 547, "Définition du genre et anatomie du génératype se révèlent donc incompatible. Par ailleurs, l'anatomie de l'espèce *nettac* Gower est celle d'un *Maritrema* typique, sans caractère d'originalité d'importance générique. . . . Il apparaît en conclusion que l'appellation *Maritreminoides* est synonyme de *Maritrema* qui est antérieure." He predicated the identity of *M. nettac* and *M. obstipum* and included the species in a new subgenus, *Atriospinosum*, in *Maritrema*.

Maritrema caridinae was described by Yamaguti and Nishimura (1944) from a gravid metacercaria found in a freshwater crustacean, *Caridina denticulata*, in Japan. Deblock (1973) considered *Maritreminoides mapaensis* Chen, 1957 a synonym of *M. caridinae*. For the species, he erected a new genus, *Quasimaritrema*, which differs from *Maritrema* only in the extend of the uterus. In the type-specimen, two loops of the uterus extend anterior to the digestive caeca. But the specimen had been crushed and the explanation of the figure notes, p. 550,

"L'écrasement à la fixation a entraîné des déformations d'organes." The validity of this genus appears to rest on a very insecure basis.

Microphallus medius was described by Van Cleave and Mueller (1932) from a single, immature, excysted metacercaria found in the intestine of *Perca flavescens*, a freshwater fish, and probably had been ingested in food. The specimen was apparently dead or moribund and was badly crushed in fixation. Mueller (1934) transferred the species to *Maritrema* and Rankin (1939) included it in *Maritreminoides*. For this specimen, Deblock (1973) proposed a new genus, *Quasimaritremopsis*, with *Q. medium* (Van Cleave and Mueller, 1932) as type-species. The generic diagnosis states, p. 554, "Même définition que le genre *Quasimaritrema* nov. gen., à l'exception de la conformation et de la disposition des glandes vitellogènes: glandes vitellogènes formées d'un petit nombre de gros follicles disposés en deux bandes transversales en avant du bord antérieur de chacun des testicules. Vitelloducts transverse probablement courts et à insertion terminale." In this specimen, on either side a loop of the uterus extends anterior to the testis to the end of the caecum.

Among other taxonomic changes, Deblock (1973) designated *Pseudospelotrema ammospizae* Hunter and Vernberg, 1953 type of a new genus, *Maritremopsis*. The diagnosis of the new genus, p. 555, states it agrees with the genus *Maritrema* Nicoll, 1907, but differs in "la conformation et la disposition des glandes vitellogènes composées d'un nombre de follicles restreint (de 5 à 10 environ), chacun d'une taille relativement volumineuse, et disposés linéairement en deux groupes situés essentiellement en avant de chacun des testicules; ils peuvent aussi s'étendre le long du contour externe de ces deux glandes. Vitelloducts transverses à insertion terminale, courts ou très courts." But in the figure of *M. ammospizae*, the vitellaria are almost identical in number, size and location with those of *Pseudospelotrema japonicum* Yamaguti, 1939, type of *Pseudospelotrema*.

The erection and suppression of genera in the subfamily *Maritrematinae* and the shuffling of species from one genus to another, illustrate the confusion and unsatisfactory taxonomic status of these parasites of migrant birds. Trivial and tenuous features have resulted in the descriptions of new species and generic concepts are indeterminate. The situation was depicted by Baer and Joyeux (1961) in the statement, p. 657, "On a décrit jusqu'à aujourd'hui plus de mille genres de Trématodes, groupés en quelque cent familles dont plusieurs monotypique. On peut cependant affirmer que cette pléthore de genres ne correspond nullement à la réalité et que leur nombre sera certainement réduit dans une proportion notable lorsque les auteurs entreprendront une révision sérieuse. On constate trop souvent que des caractères spécifique sont prétextes à créer des genres nouveaux, sans même que la limite morphologique de ceux-ci ait fait l'objet d'une investigation sérieuse. L'emploi abusif de clés dichotomique trop simples, c'est-à-dire qui sont fondées principalement sur des caractères superficiels ou variables, conduit, inévitablement vers une situation inextricable dont la conséquence est de surcharger la littérature scientifique d'une nomenclature aussi encombrante qu' inutile."

The present study has been conducted during the summer months, 1975 to 1978 at the Marine Biological Laboratory, Woods Hole, Massachusetts. Michael

DiSpezio collaborated in the work during the summer of 1975, reported in abstract, Stunkard and DiSpezio (1975). Grateful acknowledgment and special commendations are accorded John Valois, Manager of the Supply Department, for astute and discerning collections.

SUMMARY

Metacercariae, encysted in the abdominal muscles of the common prawn, *Palaeomonetes vulgaris*, taken in Quissett harbor near Woods Hole, Massachusetts, developed to maturity in recently-hatched gulls, *Larus argentatus*. The worms are identified as *Odhneria odhneri* Travassos, 1921. Sporocysts and microphallid cercariae were found in *Littorina saxatilis* at Quissett. The cercariae were ingested by small *P. vulgaris* that had been isolated in the laboratory for over two months and encysted metacercariae were recovered that proved identical with others of natural infections. Stages in the life-cycle are described. Data from seasonal infections indicate a one-year life-cycle of the parasite. Systematic and taxonomic relations of *Odhneria odhneri* are discussed.

NOTE ADDED IN PRESS

After this paper was submitted for publication, it was learned that Irene Popiel (Norwegian Journal of Zoology, 24: 303-306; figs. 1-4) had described *Cercaria littorinae saxatilis* V sp. nov. (Digenea: Microphallidae) from *Littorina saxatilis* subsp. *rudis* (Maton) in Cardigan Bay, Wales. Although somewhat larger, this larva is so similar to the cercaria of *O. odhneri* that specific identity is probable.

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MAGNETIC INFLUENCES ON PIGEON HOMING

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According to the model proposed by Kramer (1953, 1961), bird navigation is a process consisting of two steps. In the first, the bird determines its geographical position in relation to its goal and deduces the course. This step is called the "map" by analogy with human map reading. In the second, the bird selects the direction deduced in the first.

In the case of homing pigeons, a system furnishing cues about geographical position is provided by an olfactory map (Papi, 1976). The mechanisms allowing the selection of a given direction are, for these birds, of two kinds: a time-compensated sun compass and a magnetic compass (for references, see Keeton, 1974).

Many experiments designed to test the effect of magnetic forces on the orientation of homing pigeons yielded contradictory results. Keeton (1971) was the first to demonstrate clearly that the application of magnets disturbs the homing behavior of pigeons. The influence of magnets was shown to be greatest under overcast conditions, while under sun, magnets had an effect only in certain cases.

Walcott and Green (1974) confirmed Keeton's observations: using battery-powered Helmholtz coils applied to the pigeons' heads, they obtained, under overcast conditions, predictable orientation responses. In sunny conditions the effect of the coils, if present, was marginal.

As a whole, the data provided by Keeton (1971, 1972) and Walcott and Green (1974) were not consistent enough to support a conclusion that the disturbances of the magnetic field were really without effect in sunny conditions. If they had been, an interesting theoretical conclusion would have followed, namely that pigeons use one of the two compasses depending on whether the sun is visible or not, and that, in sunny conditions, the birds do not use magnetic information.

In the present study, a number of experiments were done under sunny conditions, releasing pigeons either with coils or with magnets, in order to further clarify this matter. To verify the effectiveness of the coils and to establish whether these pigeons reacted to them in the way described by Walcott and Green (1974), some tests were also performed under alternating overcast and sunny conditions.

During the preparation of the present paper, it was discovered that similar experiments had been performed independently by Walcott (1977).

MATERIALS AND METHODS

Groups of birds studied

A-birds. The first major group of birds studied consisted of 50 homing pigeons from a loft at Arnino (to the south-west of Pisa) (A1-birds). The birds were

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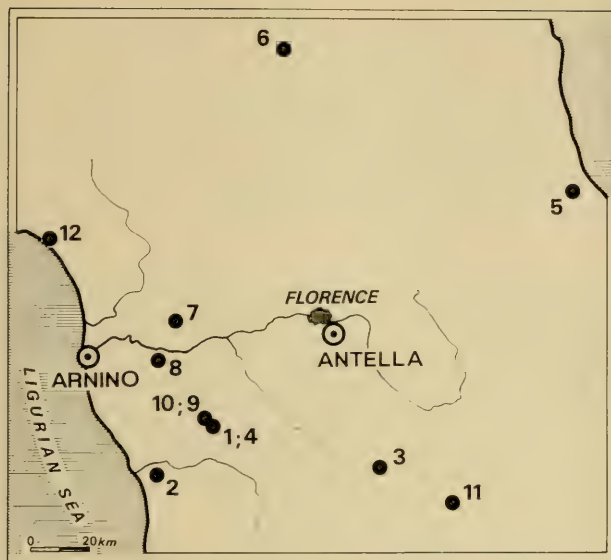


FIGURE 1. Map of the area where the experiments were conducted.

about one to two years old with flight experience to a maximum range of about 57 km N (127 km in a few cases), 133 km E, 65 km S. Before the test releases, the pigeons underwent further training releases from 14.5, 21.4, 39.3, 7.7, 39.3, 65.0, 16.4 km N, and 43.2, 51.1, 21.4, 10.2, 16.4, 31.8, 57.3, 7.7, 17.5 km S; the releases from N and S were alternated according to a random program. During the training releases, performed under both sunny and overcast conditions (8 and 9 releases, respectively), the pigeons were gradually accustomed to flying with equipment similar in size and weight to the coils used in test releases. After the training releases, since 21 A1-birds were lost, a further group of 31 pigeons (A2-birds) was added. The A2-birds had similar flight experiences to the A1-birds. This second group of birds was submitted to only two training releases from 16.4 km N and 17.5 km S under overcast and sunny conditions, respectively. During these releases, the birds wore the same equipment carried by the A1-birds during their last training releases.

In training releases, the birds were tossed singly at three minute intervals.

B-birds. The second major group of birds consisted of 48 homing pigeons from a loft at Antella (to the south-east of Florence). The birds were all about two years old and had experienced previous releases from all four compass directions, to a maximum range of about 100 kilometers (125 in a few cases). Birds were

submitted to three training releases from 21 km S, 28 and 29 km N. During training releases, two of which were performed under sunny, and one (28 km N) under overcast conditions, the pigeons were equipped and tossed in the same manner as the A-birds.

In the test releases, the same number of both A1- and A2-birds, randomly selected, were assigned to each experimental treatment. In each release with coils, the pigeons were randomly assigned to one of the three treatments. Thus, the same bird might be assigned to the same treatment more than once.

In the releases with magnets, each bird was instead exposed successively to each of the three conditions.

Coils

Helmholtz coils identically constructed and supplied as in Walcott and Green (1974) were used. This apparatus, consisting of two coils, is able, depending on the direction of flow of the current (clock-wise or counterclock-wise), to produce a magnetic field whose lines of force are directed either toward the upper coil (Nup treatment) or toward the lower coil (Sup treatment). The control birds were identically equipped, but the battery was dead and disconnected from the coils. The upper coil and the battery were secured to the pigeon's head and back, respectively, using four strips of "Velcro" tape (two tied to the apparatus, and two glued to the feathers).

Magnets

Cylindrical magnets (diameter 14 mm, height 3.8 mm, weight 2.6 g) with the poles at the bases were used. Their pole strength was approximately 450 gauss and the field strength 1.5 away from the surface was approximately 24 gauss (gaussmeter RFL mod. 750). The magnet was secured to the top of the bird's head. Depending on the polarity of the magnet directed toward the pigeon's head, there were, as with the coils, two treatments (Nup and Sup) distinguished only by the differing directions of the magnetic fields. For control, slightly smaller lead discs of the same weight were used.

Test releases

The pigeons were transported to the release site in a closed van which denied them a view of the countryside; good ventilation was ensured. Test releases were performed at unfamiliar sites (see Fig. 1). The pigeons were tossed one at a time. Nups and Sups were released alternately so that every third bird was a control. They were observed with 10 × 40 binoculars until they disappeared from sight. Bearings, vanishing times and homing performances were recorded for each pigeon.

Coils under sunny conditions

Series I: releases performed with A-birds. Release 1 was performed from Orciatice near Pisa on June 15, 1976 (9.48–12.06; clear sky, wind 2–6 m/sec from W) and on June 16, 1976 (8.39–10.47; clear sky, no wind). Release 2 was per-

formed from Bibbona near Livorno on July 1, 1976 (8.20-10.55; clear sky, wind 3-4 m/sec from E) and July 2, 1976 (8.20-9.39; clear sky, wind 2-6 m/sec from E tending to become stronger in the afternoon). Release 3 was performed from Torre a Castello near Siena on July 15, 1976 (7.48-10.10; clear sky, no wind).

Series II: releases performed with B-birds. Release 4 was performed from Orciatico near Pisa on July 4, 1977 (10.15-12.43; clear sky, wind 2-3 m/sec from N).

Release 5 was performed from Cesena near Forli on July 11, 1977 (10.30-12.47; clear sky, no wind).

Release 6 was performed from S. Giovanni in Persiceto near Bologna on August 1, 1977 (9.20-10.52; clear sky, wind 4-6 m/sec from W).

Coils under overcast conditions

Series III: releases performed with A-birds. The Release 7 was performed from Orentano near Pisa on June 24, 1976 (8.04-11.33; overcast, wind 4-6 m/sec from W).

Release 8 was performed from Fornacette near Pisa on July 20, 1976 (8.13-10.59). In this release no controls were tossed. The meteorological conditions during the experiments were unstable, with rain and partially clear intervals, during which the releases were temporarily suspended.

Release 9 was performed from Villamagna near Pisa on October 29, 1976 (10.07-12.24; overcast, wind 6-7 m/sec from E).

Magnets under sun

Series IV: releases performed with B-birds. Release 10 was performed from Villamagna near Pisa on June 3, 1977 (7.48-10.13; clear sky, wind 6-10 m/sec from N).

Release 11 was performed from Bettolle near Siena on June 9, 1977 (8.1-10.41; clear sky, no wind).

Release 12 was performed from Massa on June 24, 1977 (8.09-11.09; clear sky, no wind).

Data analysis

The circular mean for each group of vanishing bearing was calculated by vector analysis. The V-test was used to determine whether the bearings were significantly homeward clustered (Batschelet, 1972). For each release, vanishing intervals were compared by means of the Mann-Whitney U test (Siegel, 1956).

The comparison between the sets of bearings was made using Watson's U^2 test (Batschelet, 1965, 1972). Pooled vanishing intervals and homing times were compared by means of sign tests (two-tailed; Siegel, 1956).

RESULTS

Initial orientation

The results of V-test analysis are shown for each single release in Table I, and for the pooled bearings of each series under the corresponding figures (2-5).

TABLE I

The experimental results show number of birds released, and, in parentheses, of bearings recorded. The *P* values resulting from the *V*-test for the clustering around the home direction are indicated by one ($P < .05$), two ($P < .01$), or three ($P < .001$) asterisks.

Series	Release	Home distance (km)	Home direction	NUP				SUP				Controls			
				Birds released	Mean bearing	Mean vector length	V-test	Birds released	Mean bearing	Mean vector length	V-test	Birds released	Mean bearing	Mean vector length	V-test
I	1	48.3	299°	16 (12)	302°	0.71	3.48***	16 (14)	306°	0.82	4.32***	17 (15)	290°	0.71	3.85***
	2	46.2	330°	18 (15)	294°	0.83	3.70***	18 (14)	301°	0.70	3.25***	19 (19)	320°	0.92	5.57***
	3	105.0	291°	10 (10)	290°	0.74	3.29***	10 (10)	292°	0.79	3.52***	9 (7)	290°	0.97	3.64***
II	4	52.5	52°	12 (11)	47°	0.70	3.14***	14 (10)	44°	0.65	2.50**	14 (10)	56°	0.74	2.88**
	5	94.0	239°	13 (11)	285°	0.62	2.03*	12 (10)	303°	0.44	0.87	12 (11)	231°	0.35	1.61
	6	97.1	170°	7 (6)	9°	0.86	-2.86	7 (6)	311°	0.92	-2.31	7 (5)	278°	0.87	-0.65
III	7	32.0	248°	9 (9)	60°	0.85	-3.59	7 (7)	153°	0.74	-0.27	9 (8)	205°	0.31	0.92
	8	23.5	273°	12 (2)	245°	0.94	—	11 (4)	285°	0.30	—	—	—	—	—
	9	44.5	298°	10 (8)	183°	0.86	-1.45	13 (6)	296°	0.79	2.73**	8 (2)	290°	0.99	—
IV	10	52.8	57°	13 (13)	6°	0.36	1.08	13 (13)	34°	0.36	1.28	15 (14)	27°	0.70	2.57**
	11	69.7	325°	13 (13)	291°	0.41	1.74*	13 (13)	302°	0.40	1.87*	13 (13)	295°	0.94	4.20***
	12	102.0	107°	13 (8)	155°	0.40	1.08	13 (6)	344°	0.46	-0.87	13 (11)	110°	0.96	4.52***

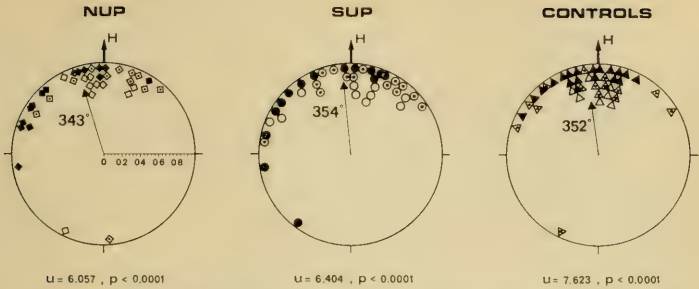


FIGURE 2. Vanishing diagrams of Nups, Sups and control birds of Series I. Bearings are pooled, setting home to 0°. In each diagram the dotted symbols correspond to Release 1, the solid to Release 2 and the open to Release 3. The inner arrow indicates the mean vector of all bearings, the length of which can be read with the scale in the first diagram.

Series I: coils under sun

The three groups were homeward oriented (Fig. 2). The differences between the pooled bearings of Sups and controls was significant ($U^2 = 0.224, P < 0.05$), whereas those between Nups and controls ($U^2 = 0.096, P > 0.10$), and between Nups and Sups ($U^2 = 0.096, P > 0.10$) were not.

Series II: coils under sun

Unlike the two other groups, Sups were not homeward oriented (Fig. 3). The vanishing bearings of Sups are not clustered around the home direction in two releases out of three, those of Nups and controls in one release out of three. Comparison between the three pooled data distributions did not show differences

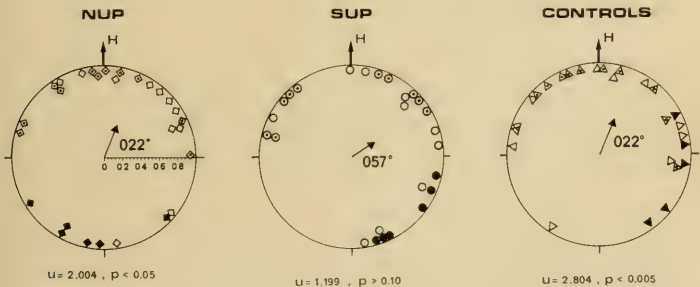


FIGURE 3. Vanishing diagrams of Nups, Sups and control birds of Series II. In each diagram the dotted symbols correspond to Release 4, the open to Release 5 and the solid to Release 6. Further explanations as in Figure 2.



FIGURE 4. Vanishing diagrams of Nups, Sups and control birds of Series III. In each diagram the open symbols correspond to Release 7, the solid to Release 8 and the dotted to Release 9. Further explanations as in Figure 2.

between Nups and controls ($U^2 = 0.029$, $P > 0.10$), Sups and controls ($U^2 = 0.064$, $P > 0.10$), and Nups and Sups ($U^2 = 0.083$, $P > 0.10$).

Series III: coils under overcast

Sups and controls were homeward oriented, whereas Nups were not (Fig. 4). Closer examination reveals that in Release 7 no group was homeward oriented, in Release 8 (without controls) recorded vanishing bearings of Sups and Nups were not enough to allow a statistical analysis and in Release 9 only Sups were homeward oriented, whereas the vanishing bearings of controls were not enough to allow a statistical analysis. Applying the V-test to pooled bearings of the Nups for clustering around the direction opposite to home, the hypothesis for randomness can be rejected ($U = 2.894$, $P < 0.005$). A different initial orientation results between Nups and Sups, as well as between Nups and controls ($U^2 = 0.355$, $P < 0.005$ and $U^2 = 0.248$, $P < 0.05$ respectively), but not between Sups and controls ($U^2 = 0.041$, $P > 0.10$).

Series IV: magnets under sun

The three groups were homeward oriented (Fig. 5). Both experimental distributions differed from those of the controls (Nups $U^2 = 0.634$, $P < 0.005$; Sups $U^2 = 0.562$, $P < 0.005$). No significant difference was noticed between Nup and Sup distributions ($U^2 = 0.037$, $P > 0.10$).

Vanishing times and homing performances

In most single releases, the vanishing intervals of the three groups were not significantly different; Nups had times longer than controls only once (Release 12, $P < 0.025$) and Sups only twice (Release 2, $P < 0.05$ and Release 12, $P < 0.01$, respectively). In Release 9, controls performed worse than Nups ($P < 0.05$). The pooled data of each series did not show any difference.

Figure 6 shows the homing times of Nups, Sups and controls in the single releases 1 through 12.

Pooling the data on homing times showed that, in Series I, Nups and Sups performed worse than controls (30 times out of 43, $z = -2.439$, $P < 0.01$ and 31 times out of 44, $z = -2.562$, $P < 0.01$, respectively); whereas no differences were observed between Nups and Sups (Nups were delayed 18 times out of 44, $z = -1.057$, $P > 0.10$). No significant differences resulted in the pooled homing performances of Series II. Since Series I and II concern releases with coils in sunny conditions, a pooling of data may be allowed. After comparing results, Nups and Sups were both seen to have worse performances than controls (48 times out of 72, $z = -2.710$, $P < 0.006$ and 48 times out of 71, $z = -2.848$, $P < 0.004$, respectively).

Data concerning homing performances of Series III as well as those of Series IV did not show significant differences between the three treatments.

DISCUSSION

The results of the experiments performed under overcast conditions (Series III) show that, in these conditions, magnetic disturbances applied to pigeons influence their initial orientation. This agrees with the results obtained by Keeton (1971, 1972), Walcott (1972) and Walcott and Green (1974). The observation that Nups oriented themselves in a direction almost opposite to that of home confirms the analogous result of Walcott and Green (1974) and further supports the theory of the magnetic orientation mechanism proposed by Wiltschko and Wiltschko (1972). The vanishing bearings of Sups and controls did not allow neat conclusions about their homeward orientation: whereas pooled bearings indicate such a tendency, a closer examination of the single releases show that Sups were homeward oriented only in one out of three releases, and controls were never homeward oriented in the two releases in which they were used. The experimental birds did not have greater difficulties than controls in reaching

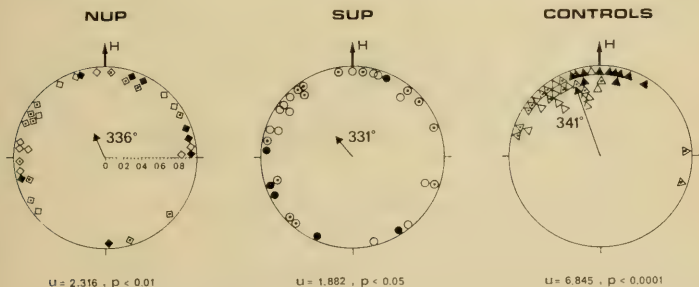


FIGURE 5. Vanishing diagrams of Nups, Sups and control birds of Series IV. In each diagram the dotted symbols correspond to Release 10, the open to Release 11 and the solid to Release 12. Further explanations as in Figure 2.

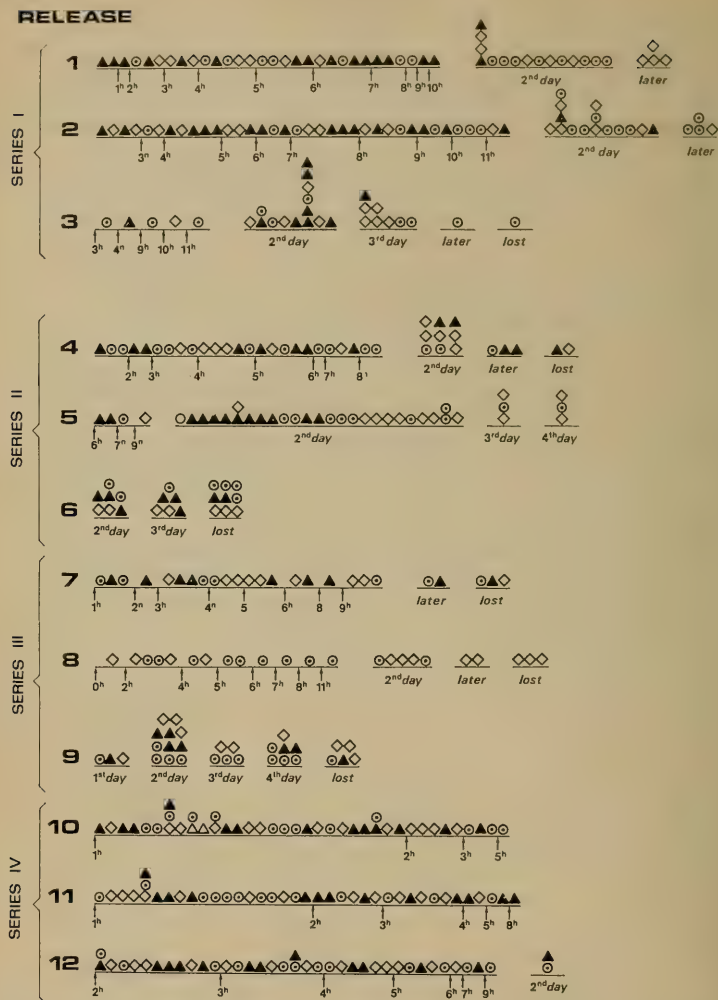


FIGURE 6. Homing performances of the Nups (open diamonds), Sups (dotted circles) and controls (solid triangles) of Series I-IV.

home. It is possible that the greater variability observed in homing performances under overcast conditions masked the effects of the experimental treatment. In any case, significant differences in homing speed were rarely observed by the above-mentioned authors.

The use of magnets in sunny conditions (Series IV) increased the scattering of vanishing bearings. The possibility that initial orientation under sun might be influenced by an artificial magnetic field had long remained uncertain, since a positive effect had only been obtained in a few experiments performed by Keeton (1971, 1972). The latest results obtained by Walcott (1977), along with the present data referring to the releases performed with magnets, give a confirmation of the phenomenon.

It may be noted that the data presented in this paper are in agreement with Walcott's observations about the directional choices of the birds wearing 0.6 gauss coils, since the mean vectors of Nups and Sups are, respectively, to the left and to the right of the mean vector for the controls.

The treatment by magnets, although causing a notable scatter in the pigeons' orientation, does not seem to cause any clear directional bias.

Pigeons released with coils under sun have in some cases significantly worse homing performances than controls. This further confirms the disturbances produced by artificial magnetic fields on homing in sunny conditions.

The data emerging from the present experiments cast doubt on the hypothesis that pigeons use a dual compass system, simply switching from a sun compass to a system based on magnetic information when sufficient sunlight is unavailable. There would seem, rather, to be an interference between magnetic information and that provided by the sun's position.

It is also possible that magnetic disturbances interfere with the map step, as suggested by recent observations by Larkin and Keeton (1976) and by Wagner (1976) concerning the influence exerted by temporal or local fluctuations of the earth's magnetic field on pigeons' orientation.

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SUMMARY

The influence of artificial magnetic fields on the homing behavior of pigeons released from unfamiliar sites was tested. The tests were performed by securing cylindrical magnets (in sunny conditions) and Helmholtz coils (under both sunny and overcast conditions) to the pigeons' heads. The apparatus had either north (Nup) or south (Sup) poles up. A distinct disorientation of Nups was observed under overcast conditions. In sunny conditions, Nup and Sup birds

showed, compared with control birds, a greater scattering of vanishing bearings and some delays in homing. The disturbances produced by an artificial magnetic field in sunny conditions demonstrate that pigeons do not simply switch between magnetic and sun compasses, but that in some way the two mechanisms interfere with each other.

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A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in WORLD LIST *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. All abbreviated components must be followed by a period, whole word components *must not* (not strictly as USASI usage, *i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.* not *J.Cell.Comp.Physiol.*)

E. We strongly recommend that more unusual words in journal titles be spelled out in full, rather than employing lengthy, peculiar "abbreviations" or new abbreviations invented by the author. For example, use *Rit Visindafjéllags Íslendinga* without abbreviation. Even in more familiar languages, *Z. Vererbungslehre* is preferred to *Z. VererbLehre* (WORLD LIST) or *Z. VererbungsL.* (USASI). *Accurate and complete communication of the reference is more important than minor savings in printing costs.*

F. All single word journal titles in full (*e.g. Veliger, Ecology; Brain*).

G. The order of abbreviated components should be the same as the word order of the complete title (*i.e. Proc. and Trans.* placed where they appear, not transposed as in some BIOLOGICAL ABSTRACTS listings).

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THE BIOLOGICAL BULLETIN

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2. **Tables, Foot-Notes, Figure Legends, etc.** Tables should be typed on separate sheets and placed after the Literature Cited. Because of the high cost of setting such material in type authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

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Continued on Cover Three

THE BIOLOGICAL BULLETIN

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CONDITIONS FOR ATTACHMENT AND DEVELOPMENT OF SINGLE CELLS RELEASED FROM MECHANICALLY-DISRUPTED THALLI OF *PRASIOLOA STIPITATA* SUHR¹

Reference: *Biol. Bull.*, **156**: 257-271. June, 1979

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Prasioola stipitata is a small thalloid green alga which grows in a rather restricted ecological niche on seagull-inhabited rocks above the tide line in northern latitudes (Lewin, 1955; Friedman, 1969). The organism reproduces asexually by means of aplanospores and sexually in a rather unusual manner where individual cells of the diploid vegetative thallus undergo meiosis to form patches of sperm and egg-bearing cells (Friedman, 1959, 1969; Cole and Akintobi, 1963). The cell wall of the organism is also unusual, consisting of inner and outer cellulose layers separated by a mucopolysaccharide matrix (Takeda, Nisizawa and Miwa, 1967; Friedman, 1959). Media have been devised on which aplanospores develop into multicellular stages leading to the mature thallus (Lewin, 1955). The present work stems from the observation that grinding of the thalli in a hand homogenizer leads not to cell breakage, but rather to the release of most of the individual cells of the thallus, attachment to a substrate and development into new plants. In this paper we describe the conditions for obtaining single cells, the physiological parameters affecting cell attachment, and the development of these single cells into the multicellular stages leading to mature plants. Brief abstracts of this work have appeared previously (Schiff, Quatrano, Harris, Legg and Staley, 1972; Bingham and Schiff, 1973; Bingham, Schiff and Ramus, 1974).

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MATERIALS AND METHODS

Thalli of *Prasiola stipitata* Suhr were collected from exposed rocks at several areas in Massachusetts in the summers of 1973 and 1974: Nobska Point near Woods Hole, Manomet Beach near Plymouth, Penikese Island, and Gay Head on Martha's Vineyard. Plants, moistened periodically with Lewin's *Prasiola* medium (Lewin, 1955), were maintained at 15° C on a 12-12 light-dark cycle. Lewin's medium was used in all experiments except where modifications are indicated. All of the plants used in any one experiment were obtained from the same collection.

Routinely, 0.5 g of *Prasiola* thalli in Lewin's medium were disrupted in either a glass or teflon hand homogenizer. Grinding was continued until most of the thalli were disrupted. Cell wall debris and non-homogenized fronds were removed either by filtration through a 100–200 μ m nylon mesh or by allowing the heavy material to settle out. The cell concentration was determined by counting in a hemocytometer, and the cells were subsequently diluted with Lewin's medium to an appropriate concentration. In most experiments and, unless otherwise noted, cell concentrations of $4\text{--}5 \times 10^5$ cells per ml were used. Fifteen ml of cell suspension were transferred to 8.5-cm petri dishes for the experiments, the details of which are given in each figure legend.

For each attachment experiment cell suspensions were gently pipetted into petri dishes 8.5 cm in diameter containing several cover slips. Attachment was assayed by removing a cover slip at an appropriate time with a forceps, and rinsing it three times in a beaker of Lewin's medium to remove unattached cells. Increased numbers of rinses did not significantly decrease the number of cells attached. The number of attached cells on the coverslip appearing in the high power field of a microscope was then counted and averaged from three randomly-selected fields as a measure of cell attachment. Counts were routinely of the order of 100 cells per field; variation in counts was of the magnitude to be expected from Poisson statistics.

To calculate the percentage of attached cells we assume that all cells contained in the cylindrical volume (V_{at}) of the petri dish above the microscope field to be counted will settle vertically; therefore the maximum number of cells that can attach will be all of those in that volume. This volume is defined as:

$V_{at} = \pi r_f^2 h$ (where V_{at} is the volume above the microscope field, r_f is the radius of the microscope field and h is the height of the cylindrical volume). (1)

The volume of the suspension added to the dish forms a cylindrical volume V_s , of radius r_d and height h :

$$V_s = \pi r_d^2 h \quad (2)$$

Solving Eq. (2) for h and substituting into Eq. (1):

$$V_{at} = \frac{r_f^2 V_s}{r_d^2} \quad (3)$$

The number of cells in the cylindrical volume V_{at} above the microscope field,

N_{af} , is related to the cell concentration, C ;

$$N_{af} = CV_{af} \quad (4)$$

Thus calculating V_{af} from Eq. (3) and substituting into Eq. (4) yields the number of cells (N_{af}) in this volume, and since this is the maximum number of cells that can attach; the number of cells actually counted in a microscope field (N_c) will be some fraction of this and can be used to compute the percent attachment:

$$N_c/N_{af} \times 100 = \% \text{ attached.} \quad (5)$$

In the experiments to be described the radius of the microscope field (r_f) was 0.0155 cm, the radius of the petri dish (r_d) was 4.25 cm, and the volume added to the dish, (V_g) was 15 cm³.

For attachment experiments petri dishes were placed at room temperature (23° C) or in 4° and 15° C cold rooms. Illumination was provided in each case by two Sylvania Cool White Fluorescent bulbs placed 18 inches above the plates providing an intensity of 140 ft-C.

In experiments where cells were incubated before attachment, cells were kept suspended at 4° in a dark refrigerator in polypropylene containers, conditions which do not permit attachment. Cells will attach to glass, polycarbonate, and polystyrene but not to polypropylene. Cell sizes were determined using a Vickers A.E.I. image splitting eyepiece and the high (43×) power objective of a microscope. The system was calibrated with a stage micrometer.

RESULTS

The structure of the cell wall of *Prasiola* is rather unusual in having inner and outer cellulose layers with a mucopolysaccharide matrix in between (Takeda *et al.*, 1967; Friedman, 1959). Dyes known to bind to acidic polysaccharides such as ruthenium red, and to sulfated polysaccharides, in particular with a meta-chromatic reaction such as toluidine blue O at very low pH, stain the matrix material, indicating that this layer is composed, at least in part, of very acidic, possibly sulfated, polysaccharides (Schiff *et al.*, 1972). When thalli of *Prasiola* are subjected to grinding in a hand homogenizer, most of the cells are released singly, leaving behind empty compartments in the thallus surrounded by the outer cell walls. The released cells rapidly attach to a substrate by means of secreted material, which appears to be composed of an acidic polysaccharide similar to the matrix material in appearance and staining properties. If these single cells are subjected to high concentrations of salt, plasmolysis can be induced allowing one to see that what was the inner cell wall in the thallus is still present as the outer cell wall of these extruded cells. These cells have the normal pigment composition of the thallus and contain chlorophyll a, chlorophyll b and carotenoids. Their rates of photosynthesis on a cell basis and chlorophyll basis are comparable to those observed for the intact thallus indicating that they have not been seriously damaged during the grinding process (Schiff *et al.*, 1972). In agreement, most of the attached cells divide to form filaments which eventually undergo division at right angles to the long axis to form the files of cells which will eventually form

the thallus. Ultimately, a thickened end representing the future thallus, a thin connecting portion which will become the stipe, and a basal portion which will become the holdfast can be seen.

Thalli of *Prasiola* normally form aplanospores at their edges. Normal vegetative cells are 7–9 μm in diameter (Fig. 1) and these enlarge to about 11–12 μm in becoming aplanospores which are then released, attach, and develop into new plants (Taylor, 1957). Figure 1 shows that grinding releases large numbers of vegetative cells and a smaller number of aplanospores. Both populations attach equally well (Fig. 1) since the distribution of sizes after grinding and after attachment are the same. Since more than 70% of the attached cells released by grinding go on to

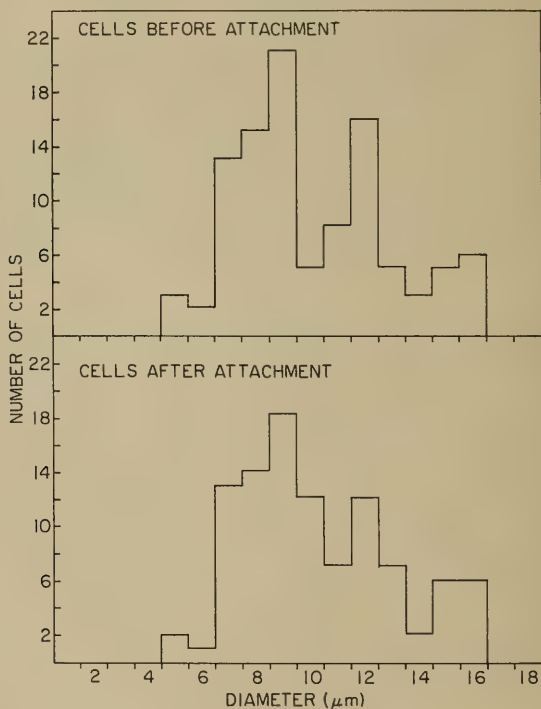


FIGURE 1. Distribution of cell sizes after release by grinding (upper) and after attachment and washing off of unattached cells (lower). Note similarity of distributions with vegetative cells peaking at 8 μm and aplanospores at 12 μm .

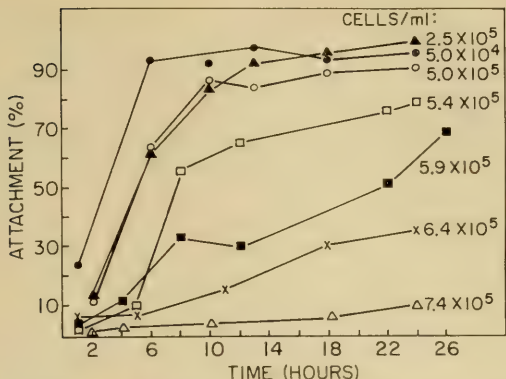


FIGURE 2. Dependence of per cent attachment on cell concentration. Cells were counted after release by grinding and were then diluted with Lewin's medium to the cell concentrations shown.

develop into plants, it is obvious that vegetative cells as well as aplanospores can develop in this manner. Indeed it appears that grinding simply converts all of the cells of the thallus into aplanospores as far as their developmental potential is concerned. Perhaps aplanospores in the thallus are simply cells at the periphery which enlarge and are capable of dissolving or rupturing their cell walls, a process we achieve in a wholesale manner through grinding.

Under normal conditions of temperature and light (Fig. 2) the degree of attachment is dependent on cell concentration. At low cell numbers (up to 5×10^5 cells ml) attachment approaches 100%. Above this concentration, the extent of attachment decreases, and instead of attaching to the substrate, the cells form clumps. Apparently at higher cell densities there is a reasonable probability that cells will collide and attach to each other rather than to the substrate. As might be expected for an organism which must survive in a rough surf, shaking the cell suspensions on a rotary shaker (160 rpm) does not alter the shape of the attachment curve nor the final extent of attachment compared with the normal assay where cells are not shaken.

The preceding experiments and most of the experiments to be described were done with cells ground and allowed to attach in Lewin's medium. Cells ground and allowed to attach in the following media were found to exhibit the same kinetics of attachment as those in Lewin's medium: Lewin's medium minus asparagine, artificial sea water (McLachlan, 1964), or natural sea water. Cells ground and allowed to attach in the following media showed little or no attachment: distilled water, NaCl (at sea water concentration, 26 g liter) or 0.1 M EDTA in Lewin's medium.

Figure 3 shows that attachment at 23° C is approximately the same in light

and in darkness. As the temperature is lowered, however, cells in the light still attach well but in the dark an inhibition of attachment is observed. The inset of Figure 3 shows the great temperature dependence of attachment in darkness compared to light. This data can be interpreted to mean that respiration or photosynthesis can provide energy for attachment. In darkness, lower temperatures would be expected to lower the respiratory rate (and the rate of other metabolic reactions). Photosynthesis in light, however, shows little effect of temperature, since the energy comes from light and since local heating can occur from energy

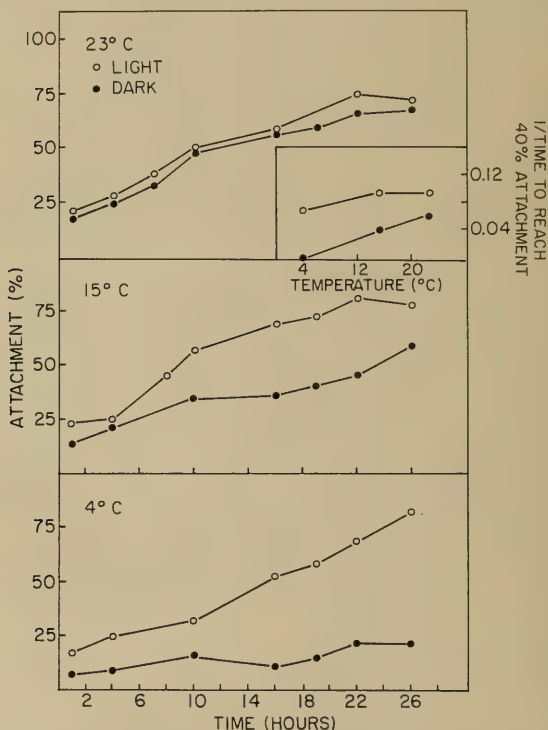


FIGURE 3. Extent of attachment in light and darkness at various temperatures. Thalli were ground at the indicated temperatures and were placed in petri dishes for the attachment assay at the indicated temperature. Duplicate dishes were placed at each temperature and one was covered with aluminum foil to darken the culture. The inset shows the reciprocal time necessary to reach 40% attachment as a function of temperature.

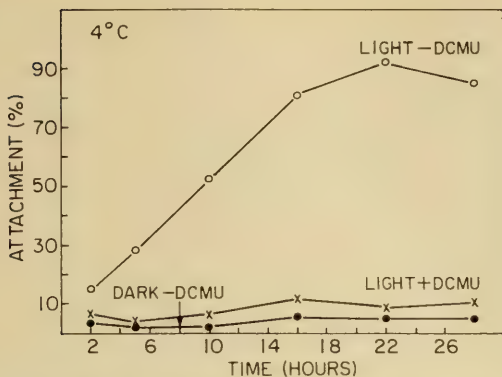


FIGURE 4. Influence of 3, (3,4 dichlorophenyl) 1,1 dimethyl urea (DCMU), an inhibitor of photosynthesis, on attachment. Cells were released by grinding at 4° C and were placed in petri dishes for the attachment assay at 4° C in the light. One dish received DCMU (final concentration 10^{-6} M) as a concentrated solution in ethanol (10^{-3} M); others received no additions, one wrapped in aluminum foil served as a dark control. Other controls (data not shown) to which ethanol of the appropriate concentration without DCMU was added in darkness and in light showed the same attachment kinetics as the corresponding controls lacking ethanol.

dissipation from the absorbed light. Thus there is little effect of temperature on attachment in the light.

To test these ideas, experiments were done with the selective inhibitor of photosynthesis DCMU which blocks the emergence of electrons from system II of photosynthesis, thereby blocking photosynthetic oxygen evolution and carbon dioxide fixation (Schiff, Zeldin and Rubman, 1967). As may be seen from the data of Figure 4, DCMU in the light at 4° C reduces attachment to the level of attachment found in darkness, indicating that most of the energy for attachment must come from photosynthesis at these low temperatures.

To test whether protein synthesis was necessary for attachment, cycloheximide, a specific inhibitor of translation on cytoplasmic ribosomes, and chloramphenicol, a specific inhibitor of translation on plastid ribosomes in most systems (Avadani and Buetow, 1972), were tested as inhibitors of attachment. In agreement with findings from other systems, addition of the antibiotics immediately before measurement resulted in little inhibition due to the permeation time necessary for the antibiotics to reach the ribosomes (Bovarnick, Chang, Schiff and Schwartzbach, 1974; Schwartzbach, Schiff and Klein, 1976). Therefore, the cells were held at 4° C in darkness to prevent attachment (Fig. 4) to allow incubation of the cells with the antibiotics for 12 hr before beginning the attachment assay. As may be seen from Figure 5, cycloheximide inhibited attachment by about 90% while chloramphenicol inhibited about 40%. This is similar to results for other plant systems where cytoplasmic and plastid protein synthesis are necessary for certain cellular

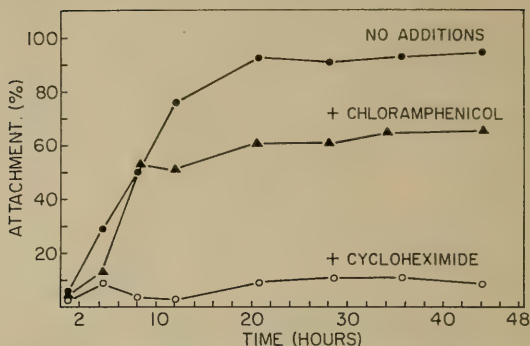


FIGURE 5. Influence of inhibitors of protein synthesis in the cytoplasm (cycloheximide) and in the plastid (chloramphenicol) on attachment. The cells were released by grinding at 4° C and were preincubated with the indicated antibiotics for 12 hr at 4° C in darkness. They were then placed at room temperature for the attachment assay.

events but cytoplasmic protein synthesis is the more important, quantitatively, of the two (Bingham, 1978). Thus both cytoplasmic and plastid protein synthesis appear to be required for cell attachment.

Metachromatically staining matrix material seems to be important for attachment, and the attached cells are seen to have mucilagenous material associated with them which stains in a manner expected for highly acidic, possibly sulfated, polysaccharides. To explore this further, experiments were performed in which sulfate was omitted from the medium or in which sulfate activation was inhibited with molybdate. Figure 6 shows that when sulfate is omitted from the attachment medium there is little inhibition of attachment. Since the cells frequently contain large sulfate stores, molybdate was added as an inhibitor of sulfate activation (Wilson and Bandurski, 1958) which is necessary for the esterification of polysaccharides with sulfate (Ramus, 1974). Since calcium molybdate is very insoluble, it was necessary to eliminate calcium from the attachment medium. Figure 6 shows that deletion of calcium had little effect on normal attachment. Addition of increasing concentrations of molybdate, however, resulted in progressively greater inhibition of attachment, complete inhibition being achieved at 0.3 M molybdate. Figure 6 also shows that sulfate added before or at the same time as molybdate relieves the inhibition to some extent, consistent with the known effect of molybdate on the ATP sulfurylase reaction as a competitive inhibitor (Wilson and Bandurski, 1958). The ATP sulfurylase reaction is the first step in sulfate activation, which forms adenosine 5' phosphosulfate (APS) from ATP and sulfate. APS is then converted to adenosine 3' phosphate 5' phosphosulfate (PAPS) by reaction with another mole of ATP in the presence of APS kinase. PAPS is the sulfate donor for all known esterification reactions involving sulfate, including the sulfation of polysaccharides (Schiff and Hodson, 1973; De Meio, 1975).

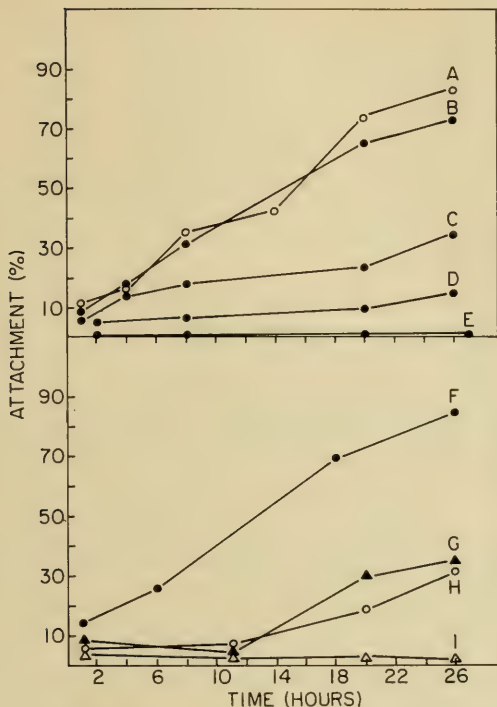


FIGURE 6. Influence of molybdate, an inhibitor of sulfate activation, on attachment. In the absence of molybdate, attachment is normal in media containing Ca^{2+} and sulfate (A) or lacking Ca^{2+} and sulfate (B). Therefore, to avoid precipitation of molybdate by Ca^{2+} , and to lower the sulfate level, Ca^{2+} and sulfate can be omitted from media used for studies of the influence of molybdate on attachment. In the absence of added Ca^{2+} and sulfate, molybdate at final concentrations of 0.01 M (C), 0.1 M (D) or 0.3 M (E) produce increasing amounts of inhibition of attachment. In the absence of added molybdate, sulfate and Ca^{2+} , once again attachment is normal (F). If 0.3 M molybdate is present during grinding and attachment, however, there is complete inhibition of attachment (I). Less inhibition is seen if sulfate is present during grinding and molybdate is added during attachment (G) or if both sulfate and molybdate are present throughout grinding and attachment (H).

Figure 7 shows the complete reversibility of the molybdate inhibition by sulfate. Cells held for various times on molybdate were then exposed to normal medium containing sulfate and were assayed for attachment. As may be seen from the data, attachment begins immediately after the cells are exposed to sulfate and proceeds normally even in cells held for prolonged periods without attachment on molybdate.

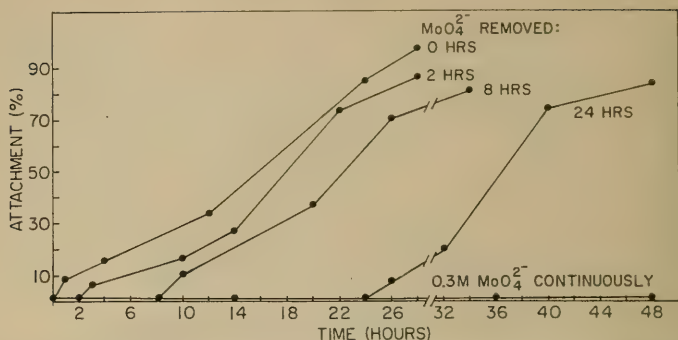


FIGURE 7. Reversal of molybdate inhibition of attachment when molybdate was removed and sulfate added after various times of incubation with molybdate. Cells were released by grinding at 4°C in a medium lacking sulfate and calcium but containing 0.3 M molybdate and were held at 4°C in darkness for the time indicated. At 0, 2, 8, and 24 hr of incubation with molybdate, an aliquot was removed, centrifuged at 121 g for 3 min at 4°C , the cells were resuspended in an equal volume of Lewin's medium and attachment was assayed at 23° as a function of time.

These experiments show that the molybdate inhibition of attachment resembles the known effects of molybdate on sulfate activation (Wilson and Bandurski, 1958; Ramus, 1974). The inhibition of both processes is competitive and is

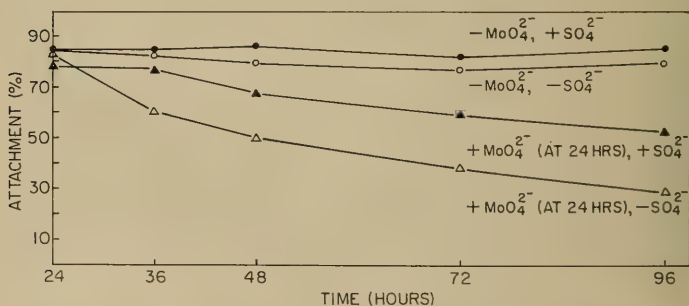


FIGURE 8. Reversal of attachment of single cells on addition of molybdate to already attached cells. All cells were allowed to attach in the normal manner. At 24 hr the medium in each of several dishes was removed and replaced with Lewin's medium (minus calcium) with additions or deletions as shown. At various times thereafter, coverslips were rinsed in the usual manner and fields counted to estimate the loss of attached cells.

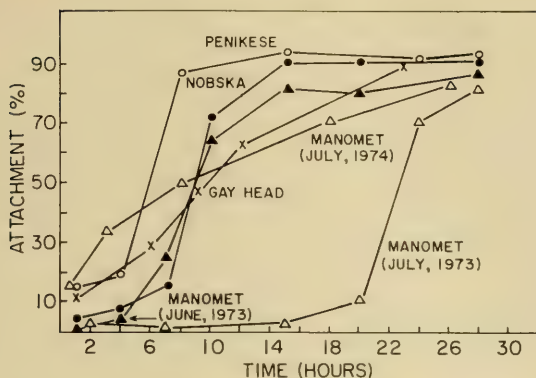


FIGURE 9. Variation of attachment kinetics with place of collection and time of collection of the *Prasiola* plants. The collection at Manomet in July, 1973 was the only one done in the rain. All cells were diluted to 5×10^5 cells/ml for the attachment assay.

relieved by addition of sulfate. Thus there is strong reason to believe that attachment requires the formation of a sulfated polysaccharide.

Figure 8 shows the influence of molybdate on cells which have already attached to the substrate. Addition of molybdate alone causes cells to disattach from the substrate. The presence of sulfate partially reverses the molybdate effect. In the absence of molybdate or sulfate the cells remain attached. The fact that attachment is reversed by molybdate indicates possible turnover in the sulfated polysaccharide thought to be necessary for attachment.

The attachment curves of cells released from *Prasiola stipitata* by grinding were not identical for material obtained from various locations at various times. Figure 9 shows attachment of single cells under constant laboratory conditions where the cells were obtained from various collections of plants. While somewhat variable, most of the collections showed good attachment within ten to twenty hrs. An exception was the material collected from Manomet in July of 1973 which attached much more slowly than the material from other collections; collection in another year at the same time showed more rapid attachment. The only variable which seems to be different is that the slow material was the only collection to be made in the rain. Since distilled water will not support attachment (see above) perhaps there was sufficient rainwater in this material to inhibit the attachment process somewhat.

DISCUSSION

It is possible to achieve nearly quantitative release of cells from thalli of *Prasiola stipitata* Suhr. by grinding in a hand homogenizer. The cells emerge

with their inner walls intact and attach to substrates by means of a mucilaginous material which is similar in staining properties to the middle wall matrix material. Since this material stains metachromatically with toluidine blue at low pH it probably contains a highly acidic, possibly sulfated, polysaccharide. The attachment process itself is most efficient at low cell numbers where the probability of cell-cell attachment is low. Attachment requires energy either from photosynthesis or from respiration. Inhibitors of protein synthesis and sulfate activation inhibit attachment, suggesting that the mucilaginous material may be composed of protein and sulfated polysaccharides, although the inhibition by inhibitors of protein synthesis could also reflect inhibition of the formation of enzymes necessary to make the materials necessary for attachment.

The involvement of protein and mucopolysaccharides in cell adhesion has been suggested for other systems (Evans, 1974). In *Entromorpha* (Evans and Christie, 1970; Christie, Evans, and Shaw 1970; Callow and Evans, 1974) and *Ulva* (Braten, 1971, 1975), green algae related to *Prasiola*, the attachment of zoospores or zygotes involves the secretion of protein-polysaccharide complexes thought to originate in the Golgi apparatus. Sulfated polysaccharides have also been implicated in attachment of *Fucus* zygotes (Crayton, Wilson, and Quatrano, 1974; Callow, Coughlan and Evans, 1978) and in the establishment of cell polarity (Quatrano, 1978) in the developing embryo. The secretion of Golgi products has also been implicated in attachment of spores of *Ectocarpus* (Baker and Evans, 1973), *Bulbochaete* (Retallack and Butler, 1972), *Polysiphonia* (Wetherbee, 1978), *Rhodella* (Evans, Callow, Percival, and Fareed, 1974), *Dictyota* (Evans and Holligan, 1972), *Laminaria*, *Pelvetia* (Evans, Simpson, Callow, 1973; Evans and Callow, 1974), and *Ceramium* (Chamberlain and Evans, 1973). Other algae such as *Euglena* (Essock, Forman, and Schiff, 1977) and *Porphyridium* (Ramus, 1972) secrete mucilages thought to contain sulfated polysaccharides and perhaps protein. The cell wall of *Chlorella* (Schiff and Hodson, 1973) contains sulfated polysaccharides thought to be deposited from Golgi vesicles. Thus the information on the involvement of sulfated polysaccharides and proteins in adhesion of single released cells from *Prasiola* is consistent with what is known about adhesion in other systems.

The observations and methodology presented in this paper open the way for further study of this interesting system. It should now be possible to study the physiology and biochemistry of the formation and secretion of sulfated polysaccharides in this well-defined system of unicells. Developmental studies should also be possible to determine the influence of cell position in the thallus on the ability of individual cells to regenerate the whole plant. Since a sexual cycle is known for this organism, it may be possible to obtain mutants affecting the formation of sulfated polysaccharides, the attachment process and the development of single cells into new plants. In short, the intact organism and the single cells released by grinding thalli of *Prasiola* offer a system in which it is possible to study the organization and development of a marine alga under controlled laboratory conditions in a kind of tissue culture where it is possible to go back and forth from the organized thallus to single cells in order to understand cell-cell relationships in a multicellular eucaryote.

This paper is affectionately dedicated to Dr. Luigi Provasoli on the occasion of his retirement.

SUMMARY

Methods are described for the nearly quantitative release of the single cells composing the thallus of *Prasiola stipitata* Suhr. by mechanical grinding in a hand homogenizer. The released cells have walls and attach to substrates quantitatively by means of a mucoid material containing acidic, possibly sulfated, polysaccharides. A simple assay for measuring attachment of these cells is described. A comparison of the size distributions of the cells immediately after grinding and after attachment indicates that both the large numbers of vegetative cells and the smaller numbers of aplanospores are attaching and developing. Normal development of the attached cells under laboratory conditions has been observed through the stage of two-dimensional division. Cell attachment is optimal up to about 5×10^5 cells per ml; higher densities are inhibitory. Low temperatures interfere with attachment in darkness but not in light; inhibition of attachment with 3, (3,4 dichlorophenyl) 1,1 dimethyl urea (DCMU) indicates that photosynthesis is necessary for attachment at low temperatures. At higher temperatures energy can probably be obtained from respiration as well. Inhibitors of protein synthesis in the cytoplasm (cycloheximide) and plastid (chloramphenicol) inhibit attachment, the inhibition by cycloheximide approaching 100%. Molybdate is a good inhibitor of attachment and is reversed by sulfate as might be expected from its action as an inhibitor of sulfate activation in the formation of sulfated polysaccharides. Cells already attached can be released from attachment by treatment with molybdate, indicating that the materials involved in attachment are subject to metabolic turnover. Algal material obtained from various places at different times show some variability in the rate of attachment but material from all collections ultimately showed complete attachment of cells.

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BIMODAL GAS EXCHANGE AND THE REGULATION OF OXYGEN UPTAKE IN HOLOTHURIANS

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Many holothurians respire bimodally, taking up oxygen both across the general body surface and through a specialized internal gas exchange system, the respiratory tree. When Winterstein capped the posterior end of *Holothuria tubulosa* in 1909, to prevent water from being pumped by the cloaca into the respiratory trees, oxygen uptake was reduced by 50 to 60%, indicating that about half of the oxygen need was met by uptake across the general body surface. Since then, bimodal respiratory capability has been studied in other holothurian species, and results have generally confirmed Winterstein's estimate of the partitioning of O₂ uptake (Choe, 1962; Newell and Courtney, 1965; Gentleman, 1970; Robertson, 1972).

Two common holothurians along the northeast coast of the United States, *Cucumaria frondosa* (Gunnerus) and *Sclerodactyla (Thyone) briareus* (Lesueur), were studied. The maximum length *C. frondosa* reaches is 50 cm, compared to 12 cm in *S. briareus* (Pawson, 1977). The comparatively few, thick, tube feet are restricted to the radii in *C. frondosa*, while they are scattered and hairlike in *S. briareus* (Pawson, 1977), numbering 2300 in an average-size animal (Colacino and Herreid, 1977).

The two species occupy quite different environments. *C. frondosa* is epifaunal and is commonly found clinging to rocks below the low water mark (Jordan, 1972). *S. briareus* occurs in muddy localities, buried or associated with eelgrass at or below the low water mark (Pawson, 1977).

Because of their differences in morphology and habitat, it might be expected that these two species would differ in the two components of bimodal respiration. Previous studies have not compared different species nor documented the response of the bimodal gas exchange system to declining oxygen partial pressure. This study also investigates the effect of body weight on the interaction and relative contributions of the two components of the gas exchange system in oxygen uptake and its regulation.

MATERIALS AND METHODS

Specimens of *Cucumaria frondosa* were collected subtidally at Lamoine Beach in Hancock County, Maine. Water temperatures in this region vary from -1.7° C in January and February to 13.9° C in August and September, with a mean salinity of approximately 32 ‰ (Jordan, 1972). *Sclerodactyla briareus* was obtained from the Supply Department, Marine Biological Laboratory, Woods Hole, Massachusetts. Animals were maintained in recirculating sea water of 30 ‰ salinity at 10° C at low densities for at least 48 hr prior to experimentation. All experiments were performed at this temperature and salinity.

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Dissolved oxygen was measured polarographically using a Yellow Springs Instrument Company model 5420 self-stirring oxygen electrode, or a model 5750 electrode, and model 54 ARC dissolved oxygen meter connected to a Linear Instruments model 161 strip-chart recorder. Individual sea cucumbers were placed in plexiglass chambers with air-saturated Millipore-filtered ($0.45\ \mu\text{m}$ pore size) sea water and the decline in dissolved oxygen was monitored continuously until cessation of detectable oxygen uptake. Different sizes of respirometry vessels were used to keep a constant ratio of biomass to volume of water for measurements made on animals of different weights. Similar experiments were performed on sea cucumbers whose cloacal openings were tied off using a loop of stout thread and further sealed with a rubber patch held in place with cyanoacrylate glue (Eastman-910 adhesive). Each animal was drained of residual sea water in the respiratory trees, slit longitudinally and drained of coelomic fluid, rinsed with distilled water, dried at 60°C and weighed. The weight-specific rate of oxygen consumption (V_{O_2}) and median P_{O_2} were subsequently calculated for each 0.5 hr interval. V_{O_2} data for both species and both treatment groups (whole animal and cloaca occluded) were plotted versus weight on a double-logarithmic scale, and regression lines were fitted by the method of least squares. Comparisons of regression line differences in slope and elevation for treatments within specific groups were performed by covariance analysis.

Mangum and Van Winkle (1973) proposed the use of the quadratic function to provide a quantitative index of the effect of P_{O_2} on V_{O_2} . The quadratic coefficient (B_2) is used as an index of oxyregulation, in which a more negative value of B_2 indicates greater regulatory capability. All measurements of median P_{O_2} and V_{O_2} over 0.5 hr intervals were commenced at a P_{O_2} of 140 mm Hg to allow the animal time to adjust to confinement and to stirring of the medium. The value of V_{O_2} at this highest P_{O_2} value was expressed as 1.0 and each subsequent value as a fraction thereof. This "normalization" of the data is required for a comparative application (Mangum and Van Winkle, 1973). B_2 values derived from quadratic curve fitting were plotted versus weight and analyzed by least squares and covariance.

Resaturation experiments were conducted in which the animals were allowed to deplete the oxygen in the chamber as previously described; then either immediately or after three hours, the water was replaced with air-saturated medium, and the experiment re-run to a second cessation of oxygen uptake.

Using an apparatus similar to that of Newell and Courtney (1965), simultaneous recordings were obtained of oxygen uptake via the respiratory trees and general body surface, and of ventilatory activity. The apparatus consisted of two chambers (310- and 260-ml), which when bolted together communicated via a hole in the central plate. Preparation of the holothurian involved mounting a rubber membrane just forward of the posterior end of the animal, using cyanoacrylate glue to insure that the membrane stayed in place and to prevent leakage. After allowing it 24 hr to adjust to the pressure of the membrane, the animal was mounted in the apparatus such that its posterior end protruded into one chamber while the bulk of the animal was contained within the other, with the rubber membrane separating the two chambers. Experimental measurements were

begun 0.5 hr later. A manometer was attached to each chamber so that pumping activity of the animal was reflected by fluid movements which were detected by a float mechanism and recorded on a kymograph. Oxygen leakage into the apparatus was prevented by sealing the manometer columns with a layer of mineral oil. P_{O_2} determinations were made using a Radiometer-Copenhagen model BMS 3 Mk 2 Blood Micro System attached to a PHM 71 Mk 2 acid-base analyzer. Periodically, 0.2 ml samples were taken through the manometer columns using a long needle and glass syringe; afterwards this same volume was returned to the chamber by injecting air-saturated sea water. A large apparatus of similar design with chamber volumes of 1500 and 530 ml was built to accommodate the larger specimens of *Cucumaria frondosa*, but was modified by having portals for oxygen electrodes to record oxygen depletion continuously. Sea water was circulated within the chambers by submersible magnetic stirrers for the smaller chambers, or by the self-stirring O_2 electrode for the larger unit. Determinations of oxygen uptake via the respiratory trees and general body surface, frequency of pumping, average volume of water moved per pumping cycle, and volume of water moved per hour were made for discrete intervals of P_{O_2} over a wide P_{O_2} range.

Oxygen extraction efficiency was determined for both species by a direct method, which involved sampling both inhalent and exhalent respiratory currents

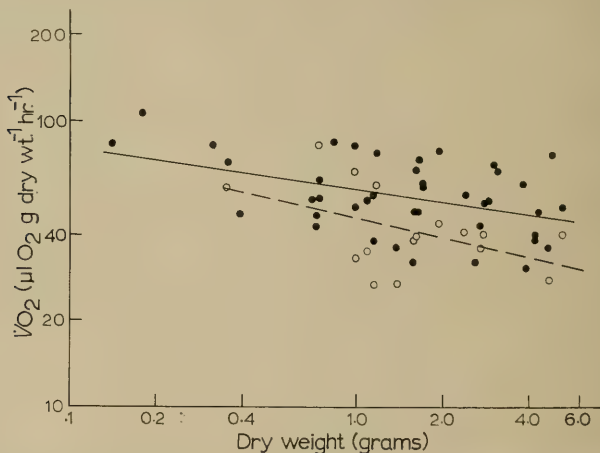


FIGURE 1. Weight-specific rates of oxygen uptake ($\dot{V}_{O_2}$) in *Sclerodactyla briareus* at 10°C as a function of body weight. Closed circles and solid line represent intact animals. Open circles and broken line represent animals with the cloacal opening occluded. Equations relating $\dot{V}_{O_2}$ to dry weight (W) for intact animals and cloaca-occluded animals, respectively, are: $\log \dot{V}_{O_2} = -0.146 \log W + 1.76$, and $\log \dot{V}_{O_2} = -0.222 \log W + 1.66$. Covariance analysis indicates that the slopes of the lines are not significantly different but that the elevations are ($0.01 > P > 0.005$).

from animals maintained in small aquaria at the experimental temperature and salinity, using a glass syringe. P_{O_2} was determined on the blood-gas analyzer. Measurement over a wide range of P_{O_2} was accomplished by bubbling the aquarium water with N_2 . Extraction efficiency was then calculated as follows:

$$\% \text{ Extraction} = \frac{P_i O_2 - P_e O_2}{P_i O_2} \times 100, \text{ where } P_i O_2 = P_{O_2} \text{ of inhaled water (mm Hg), and } P_e O_2 = P_{O_2} \text{ of exhaled water (mm Hg).}$$

A number of pH determinations was also made (again using the blood-gas analyzer) on sea water medium prior to the commencement of an oxygen consumption experiment and after oxygen uptake equaled zero. A few measurements of coelomic P_{O_2} and pH were also obtained, again before and after an oxygen consumption experiment. These were not paired measurements performed on the same animal before and after an experiment, but rather on different animals to avoid the effect of sampling, since some animals reacted by extruding a portion of the respiratory tree through the puncture.

Finally, the behavioral response to declining oxygen of three specimens of *Sclerodactyla briareus* that had been allowed to establish burrows in a sediment-filled glass bowl was examined. Periodic bubbling with N_2 of the aquarium containing the bowl produced a discrete reduction in P_{O_2} .

RESULTS

Cloacal occlusion in *Sclerodactyla briareus* results in a reduction of V_{O_2} at all body weights but no significant change in the slope of the regression line relating V_{O_2} to body weight (Fig. 1). In *Cucumaria frondosa*, on the other hand, highly

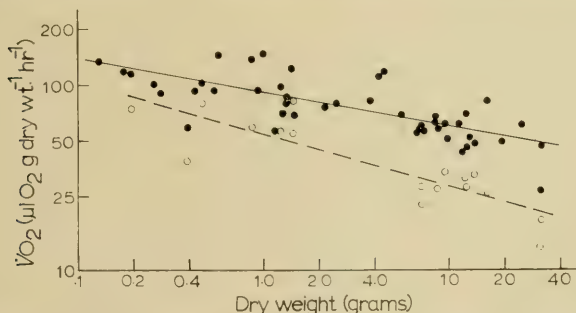


FIGURE 2. Weight-specific rates of oxygen uptake (V_{O_2}) in *Cucumaria frondosa* at $10^{\circ}C$ as a function of body weight. Closed circles and solid line represent intact animals. Open circles and broken line represent animals with the cloacal opening occluded. Equations relating V_{O_2} to dry weight (W) for intact animals and cloaca-occluded animals, respectively, are: $\log V_{O_2} = -0.182 \log W + 1.96$, and $\log V_{O_2} = -0.284 \log W + 1.73$. Covariance analysis indicates that the difference between the slopes is significant ($0.05 > P > 0.01$).

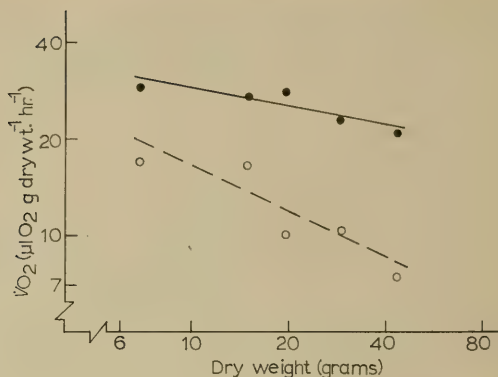


FIGURE 3. Weight-specific rate of oxygen uptake ($\dot{V}O_2$) in *Cucumaria frondosa* measured simultaneously across the general body surface and the respiratory trees, as functions of body weight. Closed circles and solid line represent respiratory tree contribution. Open circles and broken line represent general body surface contribution. Equations relating $\dot{V}O_2$ to dry weight (W) for the respiratory tree and general body surface, respectively, are: $\log \dot{V}O_2 = -0.185 \log W + 1.64$ ($r = -0.88$; $P < 0.0025$), and $\log \dot{V}O_2 = -0.472 \log W + 1.69$ ($r = -0.83$; $P < 0.01$). Covariance analysis indicates that the difference between the slopes is marginally significant ($0.10 > P > 0.05$).

significant shifts in both elevation and slope of the regression line occur (Fig. 2), indicating an increasingly important contribution to the total oxygen uptake by the respiratory trees as body weight increases in this species. This premise is further supported by Figure 3, in which oxygen uptake across the general body surface shows a greater decline as body weight increases than does oxygen uptake via the respiratory trees. No such relationship is apparent in *S. briareus*; although cloacal occlusion results in a consistent drop in $\dot{V}O_2$, no significant slope change occurs. Occlusion of the cloacal opening in *C. frondosa* results, on average, in a 45% (range 21–68%) reduction in $\dot{V}O_2$, whereas in *S. briareus*, the average reduction is only 29% (range 18–63%).

Quadratic coefficients as a function of body weight in intact animals and in animals with occluded cloacal openings are shown in Figures 4 and 5, again for both species. Both Bayne (1971) and Taylor and Brand (1975) found oxygen dependence to be an exponential relationship with weight-specific oxygen consumption ($\dot{V}O_2$) in a variety of bivalves. Such a relationship can also be found in this study (e.g., for intact *C. frondosa*, $r = 0.64$; $P < 0.001$). Because $\dot{V}O_2$ declines on a double logarithmic scale with respect to weight, and regulatory capability increases on a double logarithmic scale with decline in oxygen uptake, any relationship between regulatory ability and weight would be expected to be linear. Such is the case here. Occlusion of the cloaca affects the relationship between oxyregulatory ability (as defined by B_2) and body weight. Again, whereas

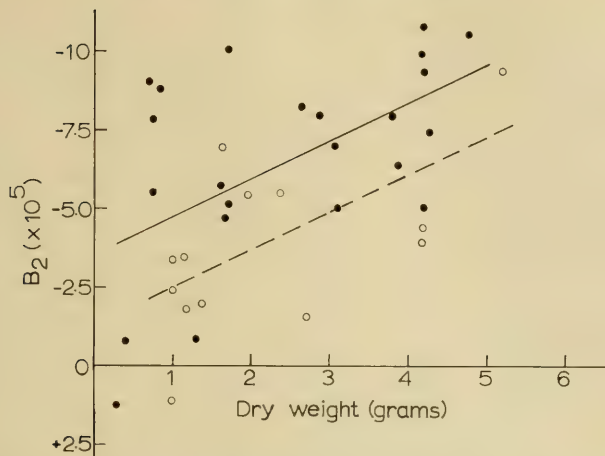


FIGURE 4. The quadratic coefficient (B_2) in *Sclerodactyla briareus* at 10°C as a function of body weight. Closed circles and solid line represent intact animals. Open circles and broken line represent animals with the cloacal opening occluded. Equations relating B_2 to dry weight (W) for intact animals and cloaca-occluded animals, respectively, are: $B_2 = -1.266W - 3.56$, and $B_2 = -1.059W - 1.42$. Covariance analysis indicates that the slopes are not significantly different, but that the elevations of the lines are ($0.05 > P > 0.01$).

both considerable slope and elevation shifts occur in oxyregulatory ability in *C. frondosa*, a significant shift in elevation alone occurs in *S. briareus*. This difference is further emphasized when specimens of both species, of similar weight, are compared for their respective abilities to maintain oxygen uptake at low P_{O_2} with their cloacal openings occluded (Table Ib). *S. briareus* not only has a higher rate of oxygen uptake at high P_{O_2} , but also maintains a greater percentage of this rate as P_{O_2} declines to intermediate levels, as compared to *C. frondosa*. These differences disappear as the P_{O_2} approaches 40 mm Hg, however.

Immediate re-exposure to air-saturated sea water of animals whose detectable oxygen consumption had ceased (resaturation experiment) produced the following results. *C. frondosa* showed a 49% increase in $\dot{V}_{O_2}$ over the normoxic state. *S. briareus*, on the other hand, did not show an increase, but a small decline of 4%. Resaturation three hours after cessation of detectable oxygen consumption elicited a markedly different response: *C. frondosa* showed a 98% increase in $\dot{V}_{O_2}$, and *S. briareus* showed a 112% increase. The F-test confirmed the homogeneity of groups with respect to weight ($P < 0.25$).

A final aspect of the respiratory response of *Sclerodactyla briareus* and *Cucumaria frondosa* to cloacal occlusion involves the cessation of detectable oxygen uptake. The average ambient P_{O_2} at which uptake ceases (residual P_{O_2}) in *C. frondosa* is significantly higher (using a simple *t*-test, $P < 0.001$) in cloaca-occluded

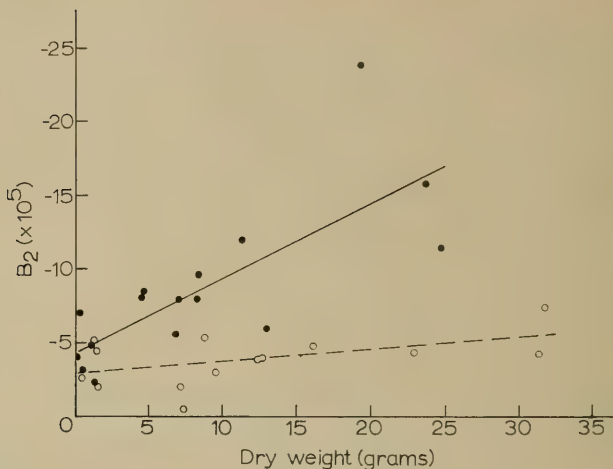


FIGURE 5. The quadratic coefficient (B_2) in *Cucumaria frondosa* at 10°C as a function of body weight. Closed circles and solid line represent intact animals. Open circles and broken line represent animals with the cloacal opening occluded. Equations relating B_2 to dry weight (W) for intact animals and cloaca-occluded animals, respectively, are: $B_2 = -0.505W - 4.29$, and $B_2 = -0.082W - 2.90$. Covariance analysis reveals that the slopes differ significantly ($P < 0.001$).

animals (5.38 mm Hg, $n = 14$) versus intact forms (3.56 mm Hg, $n = 18$). In *S. briareus*, the response is reversed, with cloaca-occluded animals showing a residual P_{O_2} of 5.10 ($n = 16$) versus 7.76 ($n = 22$) in intact animals ($P < 0.001$).

The partitioning apparatus made possible the simultaneous and independent recording of oxygen depletion of water pumped by the cloaca into the respiratory trees and that in contact with the rest of the body. In no case did leakage between the two chambers occur. This was confirmed by applying pressure to one manometer column when the animal was not pumping, and noting that the water level in the other column did not change. The results of such experiments on specimens of *C. frondosa* and *S. briareus* of similar size (6.928 and 5.474 g respectively) appear in Figure 6.

After a transitory increase in oxygen uptake across the respiratory trees in *C. frondosa*, decline in oxygen uptake via both routes is then quite rapid, leveling off at an intermediate rate at a P_{O_2} of 100–110 mm Hg (67–74% air saturation). At 50 mm Hg (33%), contribution from the respiratory trees again declines rapidly to a very low level. In *S. briareus*, oxygen uptake across the respiratory trees is consistent and continuous down to a P_{O_2} of about 65 mm Hg (44%), at which point a rapid decline in rate occurs down to a low level of about 55 mm Hg (37%). General body surface contribution, on the other hand, shows an

TABLE I

Comparison of respective abilities of individual *Cucumaria frondosa* and *Sclerodactyla briareus* to maintain oxygen uptake as P_{O_2} declines. Data for intact animals and for those with the cloacal opening occluded.

	P_{O_2} interval (mm Hg)	<i>C. frondosa</i> (wt. 4.468 g)		<i>S. briareus</i> (wt. 4.198 g)	
		$\dot{V}_{O_2}$ $\mu\text{l O}_2 \text{ g}^{-1} \text{ hr}^{-1}$	% initial $\dot{V}_{O_2}$	$\dot{V}_{O_2}$ $\mu\text{l O}_2 \text{ g}^{-1} \text{ hr}^{-1}$	% initial $\dot{V}_{O_2}$
(a) Intact animals	140-125	99.47	100	89.93	100
	100-85	76.63	77	88.52	98
	70-55	76.22	77	75.41	84
	55-40	63.49	64	65.89	73
	40-35	67.16	68	48.64	54
	35-25	53.70	54	37.40	44
	25-10	29.11	29	22.53	25
		<i>C. frondosa</i> (wt. 7.201 g)		<i>S. briareus</i> (wt. 5.207 g)	
(b) Cloacal opening occluded	140-125	29.31	100	37.60	100
	100-85	24.16	82	42.30	112
	70-55	18.03	62	31.26	83
	55-40	16.84	57	26.54	71
	40-35	15.90	54	21.13	56
	35-25	13.60	46	15.05	40
	25-10	8.00	27	9.65	26

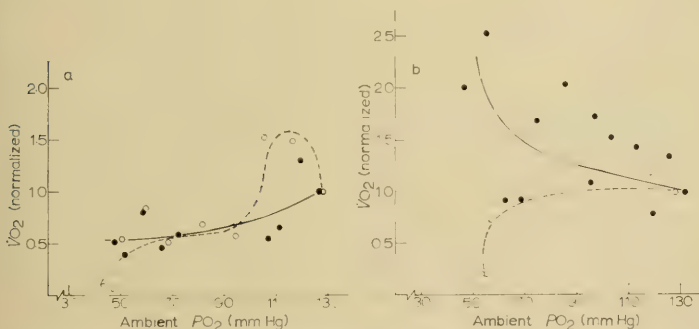


FIGURE 6. Contribution of the respiratory trees and general body surface to oxygen uptake as a function of P_{O_2} in *Cucumaria frondosa* (a) and *Sclerodactyla briareus* (b). Rates of oxygen uptake ($\dot{V}_{O_2}$) via each route are expressed as a fraction of their initial values at the highest P_{O_2} . For *C. frondosa*, the initial value for oxygen uptake via the respiratory trees is $21.82 \mu\text{l O}_2 \text{ g}^{-1} \text{ hr}^{-1}$; the initial value for uptake across the general body surface is $14.84 \mu\text{l O}_2 \text{ g}^{-1} \text{ hr}^{-1}$. The corresponding values for *S. briareus* are $22.37 \mu\text{l O}_2 \text{ g}^{-1} \text{ hr}^{-1}$ and $13.42 \mu\text{l O}_2 \text{ g}^{-1} \text{ hr}^{-1}$. Open circles and broken line represent oxygen uptake via the respiratory trees; closed circles and solid line represent general body surface contribution. Lines were fitted visually.

opposite response, with a modest increase in uptake occurring down to approximately 70 mm Hg (47%), at which point it increases sharply to a peak value at 55 mm Hg (37%).

These routes of oxygen uptake combine to produce an overall V_{O_2} pattern (again, in intact animals of similar weight) as seen in Table Ia. Broadly considered, *C. frondosa* shows an initial decline in V_{O_2} , followed by a period of consistent oxygen uptake, and then a fall-off in rate at about 35 mm Hg. *S. briareus* maintains a relatively high V_{O_2} down to about 40 mm Hg, where it begins to decline rapidly.

Simultaneous with the recording of P_{O_2} in both chambers, pumping activity was continuously monitored as the P_{O_2} declined. Since Newell and Courtney (1965) noted pumping pattern differences in *Holothuria forskali*, depending on the degree of oxygenation on the general body surface side of the apparatus versus the respiratory tree side, chamber volumes were designed such that the rate of decline within each chamber would be about the same despite differences in rate of oxygen uptake on each side.

Examples of pumping rhythms at various P_{O_2} intervals appear in Figure 7 for both *S. briareus* and *C. frondosa*. The general pattern of the pumping rhythm at high O_2 levels is similar to those recorded in other studies of holothurians (Pantin and Sawaya, 1953; Newell and Courtney, 1965; and Robertson, 1972), wherein a series of cloacal contractions causes a stepwise accumulation of water in the respiratory trees, after which a single expulsion of water occurs, completing the pumping cycle.

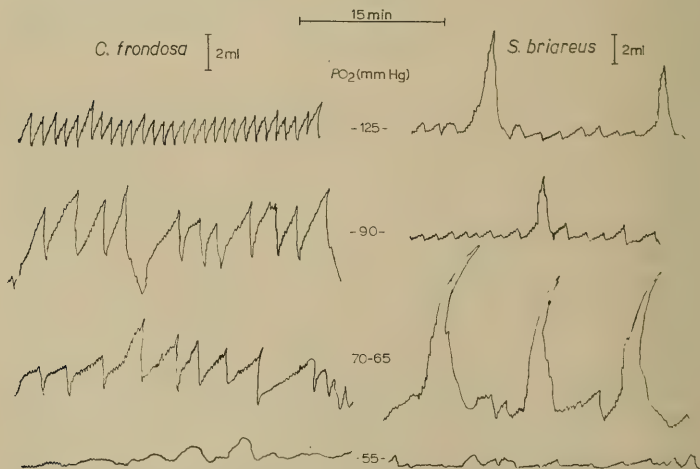


FIGURE 7. Tracings of kymographic recordings of pumping rhythms in *Cucumaria frondosa* and *Sclerodactyla briareus* at various ambient P_{O_2} s.

To summarize from Figure 7, *C. frondosa* shows a regular and continuous series of rapid pumping cycles at high P_{O_2} , changing to a lower number of much larger pumping cycles at intermediate P_{O_2} and finally entering into an erratic phase of ineffectual water movement at low P_{O_2} . *S. briareus* responds differently in that a small series of rapid pumping cycles is occasionally interspersed with a single pumping cycle of much larger amplitude at high P_{O_2} . This pattern is main-

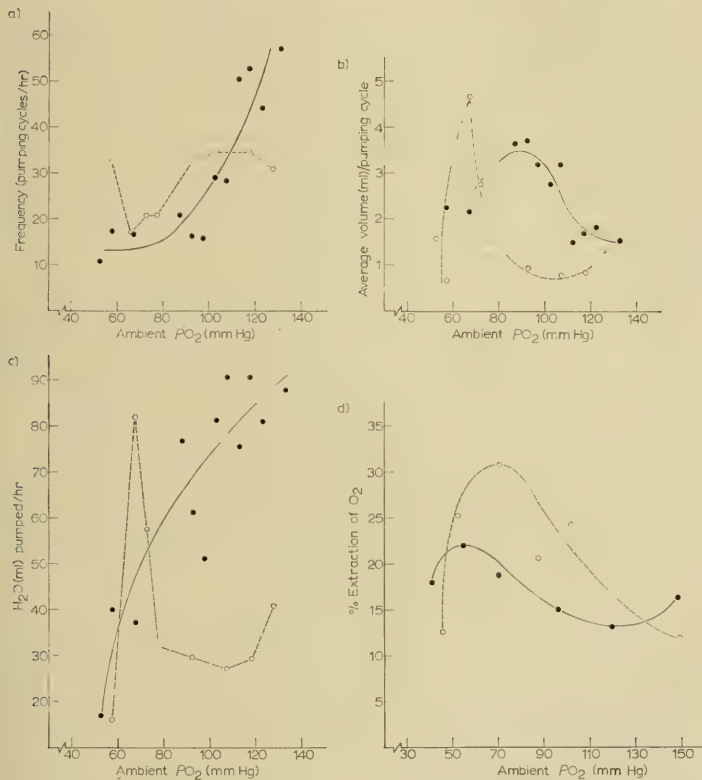


FIGURE 8. Frequency of pumping cycles (a), average volume/pumping cycle (b), and volume of water pumped/hour (c) as a function of ambient P_{O_2} , measured simultaneously (*Cucumaria frondosa*, closed circles, solid line; *Sclerodactyla briareus*, open circles, broken line). Percent extraction of oxygen (d) as a function of ambient P_{O_2} was also determined on a different pair of animals.

tained down to approximately 75 mm Hg, where the pumping pattern consists almost entirely of large peaks. Again, at low P_{O_2} , movement of water is erratic and inconsistent.

It appears qualitatively in *C. frondosa* that as P_{O_2} drops and ventilation adjusts accordingly, a "dead space" of residual water that is not being flushed out with each pumping cycle develops. No such pattern occurs in *S. briareus*.

A more quantitative comparison of the respiratory movements of the two organisms appears in Figure 8, where frequency, average volume per pumping cycle, volume pumped per hr, and per cent extraction of oxygen are each plotted versus P_{O_2} . Several differences are apparent. Both species show a decline in frequency as P_{O_2} declines, *C. frondosa* more so than *S. briareus*. Just before breakdown of the pumping rhythm, *S. briareus* shows a short-lived increase in frequency, but the amplitude of the pumping cycles is too low to affect other measurements. Lutz (1930), working on isolated cloacal circular muscle from *C. frondosa*, showed that the amount of oxygen available to the preparation modified the amplitude (augmentation at low oxygen) and the rate of onset of periodicity. Our recordings of whole animal pumping activity in *C. frondosa* are consistent with his results. Average volume per pumping cycle initially declines in *S. briareus*, then increases rapidly to a peak at 68 mm Hg. In *C. frondosa* this peak occurs at a higher P_{O_2} (90 mm Hg). Both decline rapidly thereafter. The amount of water moved per hour declines continuously in *C. frondosa* whereas in *S. briareus* steady increases occur after an initial decrease, reaching a peak at about 65 mm Hg and declining thereafter. Both *C. frondosa* and *S. briareus* show an increase in per cent extraction of oxygen as P_{O_2} declines (Fig. 11d). The extraction efficiency in *S. briareus* peaks at approximately 70 mm Hg at 31%, and in *C. frondosa* at 55 mm Hg at 22%.

Extraction efficiencies at near-saturation are 16 and 12% respectively for *C. frondosa* and *S. briareus*. No values exist in the literature for direct measurements of extraction efficiency in holothurians. Approximations of extraction efficiency can be calculated, knowing the volume of water passed in and out of the trees over a period of time, and the oxygen content at the beginning and end of this period, assuming that water is taken into the respiratory trees only once (Robertson, 1972). For *S. briareus* and *C. frondosa* in air-saturated sea water the calculated values are 42% and 35% respectively.

Finally, *S. briareus* shows a behavioral response to declining P_{O_2} when burrowed in sediment, a response which is not related to ventilation of the respiratory trees. At air saturation animals were deeply buried in the sediment, with only the extreme posterior of the animal protruding. As P_{O_2} declined, the animals began to emerge from their burrows, extending the posterior end noticeably into the water column at a P_{O_2} of 95–100 mm Hg. This emergence response continued, becoming more pronounced as P_{O_2} dropped further, such that at 40–45 mm Hg, fully 1/3 to 1/2 of the total body surface was exposed to the water column. The magnitude of the response peaked at this point since subsequent maintenance of P_{O_2} at or below this level for a period of 1.5 hr elicited no further increase in body exposure. Gradual air-resaturation of the aquarium water caused withdrawal of the animals back into their burrows.

Coelomic fluid P_{O_2} and pH measurements are given in the discussion to facilitate presentation and development of arguments.

DISCUSSION

Elimination of the respiratory tree as a functional organ of oxygen uptake by cloacal occlusion produces different results in *Cucumaria frondosa* and *Sclerodactyla briareus*. In *C. frondosa*, average reduction in $\dot{V}_{O_2}$ is considerable (45%), and changes in slope and elevation in the relationship between $\log \dot{V}_{O_2}$ and $\log$ weight indicate a decreasing ability, as weight increases, for the general body surface to compensate for the lack of respiratory trees. When oxygen uptake via the general body surface and via the respiratory trees are recorded simultaneously, an increasing contribution, as weight increases, of the trees versus the general body surface is noted. In *S. briareus*, average reduction in $\dot{V}_{O_2}$ is considerably less (30%) when the cloacal opening is blocked, and no significant slope shift occurs in the relationship between $\log \dot{V}_{O_2}$ and $\log$ weight. This indicates a greater compensatory ability of the general body surface to respond to the loss of respiratory tree contribution to oxygen uptake. One might argue that the contribution of the respiratory trees is minimal in the first place, but this is not the case. Simultaneous recordings of general body surface and respiratory tree uptake in specimens of *S. briareus* show that 59% of total oxygen uptake occurs through the trees at high P_{O_2} , and 63% in specimens of *C. frondosa* of the same weight, so the differences are minimal, at least under these experimental conditions. Furthermore, *S. briareus* is an infaunal holothurian, and the degree to which the animal may be buried is variable (see Pearse, 1908, fig. 1, p. 261). Therefore, contribution of the respiratory trees will vary depending on degree of exposure of the general body surface, because *S. briareus* apparently does not irrigate its burrow (Mangum and Van Winkle, 1973). These authors inferred a lack of burrow irrigation from the absence of an oxidized layer in the burrow in nature, and our own laboratory observations support this conclusion. Gentleman (1970) recorded differences in O_2 uptake via the respiratory trees in *Thyonella gemmata*, a common burrowing sea cucumber, depending on the degree of oxygenation of the general body surface, which had a different response than the respiratory trees in O_2 uptake as P_{O_2} declined.

Elimination of the respiratory trees from participation in oxygen uptake has an effect on regulatory ability similar to that seen for $\dot{V}_{O_2}$. In *C. frondosa*, regulatory ability is reduced dramatically (average 55% decline in B_2) and the effect is more pronounced with increasing weight, as indicated by a significant slope change relative to intact animals. In *S. briareus*, no significant slope change occurs but an elevation change does take place, resulting in a 42% reduction in the B_2 value on average. Indications are, therefore, that the general body surface of *S. briareus* can better cope with the loss of the respiratory trees in maintaining regulatory ability, which implies a role for the body surface in oxyregulation in intact specimens. Some further support of this argument is the fact that specimens of *S. briareus* with their cloacas occluded have a lower residual P_{O_2} relative to intact animals, whereas *C. frondosa* shows the opposite.

Although Mangum and Van Winkle (1973) predicted a severely reduced O_2 permeability of the muscular body wall of *S. briareus*, they did not comment on

the importance of the tube foot in gas exchange in this species. Recently, Colacino and Herreid (1977) made calculations, based on the oxygen-binding characteristics of hemoglobin in the water vascular system and the counter-current flow pattern and velocities of fluid streams in the tube foot, that most of the oxygen transport in *S. briareus* in air-saturated sea water is via the tube feet. They likely overestimated the importance of this transport system because *S. briareus* in nature occupies a non-irrigated burrow. Oxygen uptake by the tube feet is undoubtedly important when the animal extends its body into the water column, a behavior elicited by declining P_{O_2} . Under conditions of declining P_{O_2} then, the role in oxygen uptake of the tube feet, which contain a high affinity hemoglobin (Colacino, 1973), could be enhanced, and their increasing participation in oxygen uptake is indeed implicit in Figure 6b.

Dimock (1977) found that the loss of the respiratory trees in eviscerated specimens of *Stichopus parvimensis* did not affect the pattern or magnitude of weight-specific oxygen consumption relative to that of intact animals. However, he made no mention of whether mean $\dot{V}_{O_2}$ values and values of the oxygen dependence index (K_1/K_2 of Bayne, 1971) for eviscerated and intact animals were developed from groups homogeneous in weight. Further, although Dimock (1977) stated that no change occurred in the pattern of oxygen uptake in eviscerated versus intact animals, the eviscerated animals had in fact a 28% increase in the oxygen dependence index, indicating an increase in oxyconformity. Further, the critical P_{O_2} shifted from 50 mm Hg to 75 mm Hg.

Individuals of *C. frondosa* and *S. briareus* of similar weight differ in their abilities to maintain $\dot{V}_{O_2}$ as P_{O_2} declines (Table I). In both intact and cloaca-occluded animals, *S. briareus* exceeds *C. frondosa* in regulatory capability. The superior ability of *S. briareus* probably results from a greater capability to take up oxygen across the general body surface. Also, *S. briareus* can extract more oxygen and pump more water through the respiratory trees as P_{O_2} declines. In *C. frondosa*, extraction efficiency increases some, but the amount of water pumped declines with P_{O_2} . Thus, as ambient P_{O_2} declines, oxygen uptake via the respiratory trees is maintained at a consistently higher rate relative to the initial rate at the highest P_{O_2} in *S. briareus*, as opposed to *C. frondosa*. In addition, the uptake of O_2 across the general body surface in *S. briareus* increases in response to any decline in transport via the respiratory trees as P_{O_2} falls. Such an observation is again supportive of the responsiveness which the general body surface has shown in previous experiments. Much of the total oxygen requirement of *S. briareus* at high P_{O_2} can be satisfied by either respiratory system. Steen (1965) found in the sea urchin *Strongylocentrotus droebachiensis* that the calculated capacity for O_2 uptake (based on tube foot dimensions and P_{O_2} gradients) far exceeded the actual $\dot{V}_{O_2}$ levels measured, and attributed this to a poorly developed circulatory system lacking a respiratory pigment. In the case of *S. briareus* "excess" respiratory capacity appears to be involved in the maintenance of high $\dot{V}_{O_2}$ levels under hypoxic conditions.

We note that the calculated oxygen extraction efficiencies in *S. briareus* and *C. frondosa*, respectively, are 42 and 35% at air-saturation, in the same range as those reported for *Holothuria forskali* (49%; Newell and Courtney, 1965) and

Stichopus mollis (55%; Robertson, 1972). However, our *direct* determinations of extraction efficiencies are substantially lower (12% in *S. briareus*; 16% in *C. frondosa*). This indicates that the criterion of the indirect method, that water be taken into the respiratory trees only once, is not met in closed-vessel experiments.

S. briareus produces lactate as a major glycolytic end-product, lactate production occurring in those species with only a moderate degree of tolerance to anoxia (Ellington and Hammen, 1977). Highly tolerant species produce end-products other than lactate, such as succinate and volatile fatty acids, the advantages of which have been noted in Hochachka, Fields and Mustafa (1973) and de Zwaan (1977). It would therefore seem advantageous to *S. briareus* to regulate oxygen uptake in order to maintain aerobic metabolism as oxygen declines, particularly since it occupies an environment where repeated hypoxic exposure is likely.

The significance of the greater regulatory ability of *S. briareus* relative to *C. frondosa* is apparent in the resaturation experiments. Whereas *S. briareus* showed no $\dot{V}_{O_2}$ increase upon resaturation after oxygen consumption ceased, *C. frondosa* had a considerable increase. Both species responded to resaturation three hours after oxygen consumption ceased with an increase in $\dot{V}_{O_2}$. The pH values of ambient sea water at 10° C before and after oxygen was depleted from the respiratory chamber show little difference between the two species (from 7.43 to 7.08 for *C. frondosa* and from 7.42 to 7.06 for *S. briareus*). Changes in coelomic fluid pH also show a similar pattern (from 7.06 to 7.04 for *C. frondosa* and 7.11 to 7.05 for *S. briareus*). The similarity of these changes in the two species may indicate no substantial difference in the magnitude of release of anaerobic end-products.

At 140 mm Hg external P_{O_2} , coelomic fluid P_{O_2} for *S. briareus* is 100 mm Hg (Mangum and Van Winkle, 1973) versus 102 mm Hg ($N = 4$) for *C. frondosa* (this study). At cessation of oxygen uptake, coelomic fluid P_{O_2} in *S. briareus* is 62 mm Hg (Mangum and Van Winkle, 1973) and that of *C. frondosa* is 23 mm Hg ($N = 3$) (this study). By avoiding an oxygen debt and maintaining a high internal P_{O_2} at low ambient P_{O_2} , *S. briareus* seems to be more adapted to cope with hypoxic conditions, especially if oxygen stored in the coelomic fluid maintains some degree of aerobic metabolism after external oxygen uptake has ceased.

An "oxygen debt" response could mean a number of things in holothurians. An increase in $\dot{V}_{O_2}$ could be caused by increasing activity required for flushing acidic metabolites (C. P. Mangum, personal communication to Ellington and Hammen, 1977), re-oxygenation of oxygen-depleted coelomic reserves (Newell and Courtney, 1965), reoxidation of anaerobic end-products, or any combination of the three. Needed are measurements of internal oxygen partial pressures and mechanisms of anaerobiosis, particularly in *C. frondosa*.

A number of physiological capabilities have been attributed to organisms possessing bimodal respiratory surfaces. One obvious example is the ability to breathe in two media. Such a capability has little relevance to most holothurians. However, a number of recent works on amphibious salamanders has contributed to the understanding of bimodal respiration. Whereas bimodal respiration (via the lungs and skin) allowed increased metabolic rates in large salamanders in

hypoxic and/or aquatic conditions (Beckenbach, 1975; Ultsch, 1976), no significant differences between slopes of standard metabolism versus body weight were found in lunged (bimodal) versus lungless (unimodal) salamanders in air (Feder, 1976). Rates of oxygen uptake in holothurians with respiratory trees have not been compared to those without trees (order Apodida) at high P_{O_2} levels, although examination of the oxyregulatory ability of *S. briareus* in this study, and in Mangum and Van Winkle (1973) indicates greater regulatory capability when compared to *Leptosynapta inherens* (Mangum and Van Winkle, 1973), which lacks both respiratory trees and hemoglobin.

There are nevertheless examples of bimodally respiring sea cucumbers which do not have a high degree of oxygen independence. One is *C. frondosa*. Although large individuals of this species show very high regulatory capability, smaller specimens of similar weight to *S. briareus* show less capability. This could be due to the increasing role the respiratory trees play in supplying total oxygen needs in large *C. frondosa* in conjunction with the critical importance of the tree in oxyregulation in large specimens (Fig. 5). Other examples include *Holothuria forskali* (Newell and Courtney, 1965), *Stichopus parvimensis* (Dimock, 1977) and *Paracaudina* sp. (Nomura, 1926, cited by Newell and Courtney, 1965), all of which show a dependence on the P_{O_2} of the surrounding medium with respect to oxygen uptake. It is apparent, particularly in the last three examples given, that the bimodal capability must be functioning in some role other than strictly that of oxyregulation. Feder (1977) concluded that the extra respiratory surface area in bimodally respiring salamanders allows for a more rapid repayment of oxygen debt following activity. Holothurians show a considerable oxygen debt response, and whether this debt is metabolic or systematic, rapid repayment would presumably be advantageous.

It appears then, that in response to declining oxygen partial pressure, bimodally respiring holothurians may use an internal oxygen store (Newell and Courtney, 1965), oxyregulation, or a combination of the two. The strategy may depend on the frequency and duration of hypoxia routinely encountered. *Cucumaria frondosa* and *Holothuria forskali* are found subtidally on rocky bottoms (Jordan, 1972; Newell and Courtney, 1965) where exposure to hypoxic conditions would probably be only occasional. Such encounters would involve incurring an oxygen debt.

Sclerodactyla briareus, on the other hand, is found subtidally in shallow water, usually buried in muddy substrates, or associated with eelgrass beds (Pawson, 1977). Under these conditions, exposure to hypoxic conditions might be expected to be more frequent. Continuous incurrence of oxygen debts could be energetically costly to *S. briareus* for reasons mentioned earlier. As an alternative, an effective bimodal system of oxygen uptake has evolved that maintains uptake as P_{O_2} declines and eliminates the necessity of an oxygen debt response unless exposed on a long term basis to low oxygen levels.

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SUMMARY

In air-saturated sea water, 60% of the total O_2 uptake by *Cucumaria frondosa* (epifaunal) and *Sclerodactyla briareus* (infaunal) occurs through the respiratory trees. When the cloacal opening of *C. frondosa* is occluded, weight-specific O_2 uptake decreases with weight more than in intact specimens, indicating an increasing dependence of large individuals on their respiratory trees. Likewise, cloaca occlusion results in a proportionately greater loss of oxyregulatory ability in large *C. frondosa*. Neither of these results occurs in *S. briareus*, which compensates for loss of the trees by increasing O_2 uptake across the general body surface.

In intact animals, the greater oxyregulatory ability of *S. briareus* relative to *C. frondosa* is also due to the former's having less of a decline in frequency combined with a more pronounced increase in volume per pumping cycle of the respiratory tree irrigation rhythm, resulting in an increase in total volume of water pumped, as P_{O_2} declines; in *C. frondosa*, this volume declines continuously with P_{O_2} . Also, a greater increase in extraction efficiency occurs in *S. briareus* as P_{O_2} declines. Such differences between these species represent adaptation to habitats differing in availability and variability of O_2 .

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TOXICITY AND PHARMACOLOGY OF EXTRACTS FROM DORID NUDIBRANCHS

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The delicate, shell-less nudibranch mollusks are widely distributed in the seas of the world, where they survive without the benefit of the armor of most other mollusks. The mechanisms of defense among members of this order are extremely varied. Most members of the suborder Aeolidacea feed on coelenterates and are able to ingest and store the nematocysts of their prey in cnidosacs in the tip of their dorsal cerata (Edmunds, 1966; Harris, 1973). These nematocysts contain a variety of toxins (Picken and Skaer, 1966; Goodwin and Telford, 1971) that presumably are used for defense. Members of the suborder Doridacea, usually associated with sponges, bryozoans, and tunicates (MacBeth, 1971; Thompson, 1976; Harris, 1973) must rely on other defenses. Some exhibit swimming or similar escape behavior (Willows, 1969; Edmunds, 1968a; Farmer, 1970; Wobber, 1970), others incorporate calcareous spicules into their tissues, perhaps decreasing their attractiveness as food (Thompson, 1960; Paine, 1963). Some have warning coloration.

Since the dorid nudibranchs generally have few predators (cf. Harris, 1973) it might be reasoned by analogy to other marine invertebrates (Ciereszko, 1970) that they possess some chemical means of defense. However, we are aware of only two examples of the presence of noxious chemicals in members of this suborder. Certain of them secrete sulfuric acid (Edmunds, 1966; 1968b). One of them, *Phyllidia varicosa*, emits a smelly mucus, toxic to marine animals (Johannes, 1963). The active substance is a sesquiterpenoid isocyanide that was isolated from *Phyllidia* and from the sponge (*Hymeniacidon*) on which it feeds (Burreson, Scheuer, Finer, and Clardy, 1975).

We report here that toxic substances occur in California dorid nudibranchs. Eolids have not been included because of the obvious problem posed by the presence of nematocysts during chemical separation of their toxins from those possibly present in the nudibranch itself. Extracts of dorids have characteristic and reproducible effects in vertebrates and in isolated tissue preparations. Detailed reports of the chemistry and pharmacology of the substances concerned will be reported elsewhere.

MATERIALS AND METHODS

Nudibranchs

The animals were collected intertidally from 1967 to 1978, and subtidally since 1974 in the vicinity of Monterey Bay, California. Except where specifically noted they were kept in aerated aquaria with running sea water at 14° to 19° C for 2

to 20 days before use in experiments. Some were then frozen and stored at -20°C until required. Because of their availability, most work was done with specimens of *Anisodoris nobilis* MacFarland, 1905 and *Doriopsisilla albopunctata* Cooper, 1863, which is synonymous with *Doriopsis fulva* MacFarland, 1905, *Dendrodoris fulva* MacFarland, 1905, and *Dendrodoris albopunctata* (cf. Steinberg, 1961; Roller, 1970).

Preparation of tissue extracts

For determination of toxicity, the fresh or frozen animals or their tissues were homogenized in a Waring Blendor in an equal volume of distilled water. The homogenates were then treated in one of two ways. Supernatant solutions were prepared by centrifugation at 0°C and $10,000 \times g$ for 10 min. Dialyzates were prepared by dialyzing against distilled water at 5 to 7°C through Spectrapor membrane tubing No. 2 (MW cutoff 12,000–14,000) or No. 1 (MW cutoff 6000–8000). A rotating dialyzer was used and fluid was changed every 12 hr, 2 to 6 times total. Supernatant solutions and dialyzates were concentrated as necessary by lyophilization.

Toxicity

The toxicity of various tissue extracts was determined by injection into mice or crabs. Volumes of 0.1 to 1.0 ml were injected intraperitoneally into adult white mice weighing 20 to 30 g. Volumes of less than 0.5 ml were injected into the hemocoel of shore crabs (*Pachygrapsis* or *Hemigrapsis*) at the base of the largest walking leg.

Pharmacological preparations

The frog rectus abdominis muscle preparation of Chang and Gaddum (1933) was cut from small specimens of *Rana catesbiana*. It was suspended in aerated frog Ringer solution at room temperature and connected to a displacement transducer (Phipps and Bird Model ST-2). Guinea pig ileum was cut into 2 cm portions, secured at one end to an L-rod and at the other to the transducer mentioned above and suspended in an aerated bath of Tyrode's solution at 37°C . Guinea pig atria were freed from ventricular tissue, mounted in a bath of Krebs (1950) bicarbonate Ringer gassed with 5% CO_2 to 95% O_2 at 30°C and connected to a Grass FT 0.03 force transducer. In all instances records were obtained on a Grass Model 5A Polygraph. In general the methods followed for isolated preparations were those of the Department of Pharmacology of the University of Edinburgh (Perry, 1969). Very large male rats were anesthetized with sodium pentobarbital and the arterial pressure was recorded from a cannula inserted into one carotid artery and connected to a Statham Model P23AC transducer. Injections were made into the femoral vein.

Chemical methods

Dialyzates prepared as above were chromatographed on 0.5×27 cm or 0.9×20 cm columns of Sephadex G-10 or Biogel P-2. In all cases the void volume was

determined with Blue Dextran and the fractions were eluted with distilled water at a rate of approximately 0.3 to 1.0 ml/min. Ascending chromatograms were run on 10×26 cm sheets of Whatman #1 paper at 20° C using n-butanol:acetic acid:water (12:3:5). Detection reagents were aqueous diazotized sulfanilic acid (Pauly Reagent) followed by Na_2CO_3 (Erspamer, Vitali and Roseghini, 1964) or aqueous bismuth subnitrate, acetic acid, and KI (Dragendorff reagent). Histamine was determined by the spectrofluorometric method of Shore (1971).

RESULTS

Toxicity

Simple extracts (see Methods) of whole nudibranchs or of their digestive glands were injected intraperitoneally into mice with the results shown in Table I. It is clear that extracts of *Cadlina flavomaculata*, *Doriopsilla albopunctata*, *Anisodoris nobilis*, *Archidoris montereyensis* and *A. odhneri* are toxic. It may be significant that all three species of the superfamily Phanerobranchia studied were not toxic.

Although evidence of toxicity by intraperitoneal injection into mice is a standard method for pharmacology, we have also determined the toxicity of extracts of nudibranch tissues in shore crabs. Supernatant solutions prepared from the digestive glands of specimens of *Anisodoris nobilis*, *Doriopsilla albopunctata*, *Cadlina flavomaculata*, *Archidoris montereyensis*, and *A. odhneri* injected into the hemocoel of specimens of *Pachygrapsis* or *Hemigrapsis* in doses of 1.7 g/kg proved to be lethal within 2.5 hr. In addition, similar extracts of specimens of *Diaulula sandiegensis* that were not toxic to mice in doses up to 13 g/kg whole dorid, were toxic to crabs in about 20 hr in a dose of 1.7 g/kg of digestive gland. Probably the difference in toxicity in mice and crabs is a function of dose as well as species.

Localization of the toxic factors

In several experiments the digestive gland, mantle and foot of specimens of *Doriopsilla albopunctata* were separately homogenized and the supernatant solution assayed for toxicity. Only extracts of the digestive gland were found to be toxic. It must be emphasized that lethality in mice is a relatively crude measure of biological activity, and these experiments by no means exclude the presence of the kind of biologically active substances found by others in nervous tissues from other gastropods (cf. Greenberg, Agarwal and Wilkins, 1973). Using supernatant solutions of the digestive glands of specimens of *D. albopunctata*, the survival time of mice decreases with increase in dosage, as is to be expected. For a dose equivalent to 25 g/kg fresh digestive gland the survival time was 106 min in three experiments. At 50 g/kg it was 75 min and at 100 g/kg it was 18 min.

Identification of pharmacologically active substances

We could not find any evidence (using indicators) that these nudibranchs secrete acid, even when disturbed.

TABLE I

Toxicity of extracts of nudibranchs in mice. Extracts were injected intraperitoneally into mice. The dose is the equivalent weight of fresh tissue injected per kg mouse weight.

Nudibranch species	Tissue	Dose (g/kg)	Number of mice		Time to death (hours)
			Injected	Dead	
<i>Anisodoris nobilis</i> (MacFarland, 1905)	Whole	5.0	3	0	—
		10.0	4	1	60
		20.0	4	3	36
	Digestive Gland	40.0	2	2	4
		50.0	4	4	21
<i>Archidoris montereyensis</i> (Cooper, 1862)	Whole	20.0	2	2	48-72
<i>Archidoris odhneri</i> (MacFarland, 1966)	Digestive Gland	1.6	4	4	3-5
<i>Cadlina flavomaculata</i> (MacFarland, 1905)	Whole	2.0	2	1	60
		10.0	2	2	12-36
		20.0	4	2	12
<i>Diaulula sandiegensis</i> (Cooper, 1862)	Whole	5.0	2	0	—
		7.0	2	0	—
		13.0	2	0	—
<i>Doriopsilla albopunctata</i> (Cooper, 1863)	Whole	10.0	5	3	48-72
		20.0	3	3	24
		42.0	1	1	12
	Digestive Gland	10.0	2	0	—
		15.0	2	1	1.25
		25.0	2	2	1.77
		50.0	2	2	1.25
		100.0	1	1	0.3
<i>Hopkinsia rosacea</i> (MacFarland, 1905)	Whole	10.0	1	0	—
		30.0	1	0	—
<i>Hypselodoris californiensis</i> (Bergh, 1879)	Viscera	2.0	1	0	—
<i>Triopha carpenteri</i> (Stearns, 1873)	Whole	10.0	1	0	—
		20.0	1	0	—
		42.0	2	0	—
<i>Triopha maculata</i> (MacFarland, 1905)	Whole	2.0	1	0	—
		10.0	1	0	—
		26.0	1	0	—

Dialyzed extracts of the digestive glands of specimens of *Doriopsilla albopunctata* and *Anisodoris nobilis* were chromatographed on paper together with standard solutions of histamine, serotonin (5-hydroxytryptamine), tetramine, and octopamine. Dialyzed extracts of the digestive glands give primarily two spots: one with an $R_f = 0.44$ was orange-red with the Pauly reagent and is unidentified. The other, with an $R_f = 0.30$, was orange-red with the Pauly reagent and was probably due to histamine. Histamine was determined photofluorimetrically in extracts of the digestive gland of *Doriopsilla albopunctata* and *Anisodoris nobilis* extracted with perchloric acid as for brain tissue according to Shore (1971). The mean histamine content of eleven samples was $5.2 \mu\text{g/g}$ fresh tissue as histamine base.

Various extracts of the digestive glands of all the dorid nudibranchs found toxic in mice (Table I) were tested for pharmacological activity on the isolated frog rectus abdominis muscle and on the isolated guinea pig ileum. The frog rectus abdominis is a standard preparation for the assay of acetylcholine and related choline esters (Whittaker, 1963). Supernatant solutions of the digestive glands of the five species found to be toxic in mice were assayed on this muscle. In no case was a contraction observed at concentrations equivalent to 1.4 g/l of fresh gland. Standard solutions of acetylcholine perchlorate produced contractions in concentrations of $1.67 \mu\text{g/l}$. Thus, the extracts of digestive glands of the dorid nudibranchs tested contained less than $1.2 \mu\text{g/g}$ acetylcholine.

Dialyzates of the digestive glands of specimens of *Doriopsilla albopunctata* were tested for biological activity on the isolated guinea pig ileum (60 ml tissue bath). Contractions were produced by dialyzates representing approximately 400 μg of fresh gland. The contraction produced by the dialyze was not blocked by atropine (1 μg), an antagonist of acetylcholine, thereby confirming the results obtained on the rectus abdominis muscle that these dialyzates do not contain acetylcholine. The contractions were also not blocked by D-2-bromolysergic acid diethylamide (BOL-148) (10 μg), a serotonin inhibitor, indicating that the contraction is not caused by serotonin. However, the contraction was partially (but not completely) blocked by the histamine inhibitor tripeleannamine (Pyribenzamine) (5 μg), a dose that completely blocked the effect of 0.2 μg histamine (Fig. 1). From a plot of percent maximal contraction of the ileum vs. log concentration of histamine it was estimated that 1 g fresh digestive gland contained an equivalent of 916 μg histamine dihydrochloride.

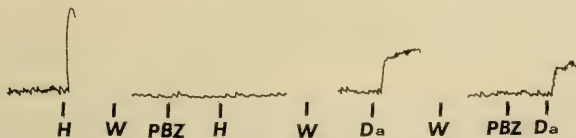


FIGURE 1. Effect of dialyze of the digestive gland of *Doriopsilla albopunctata* on contraction of the guinea pig ileum. All amounts were added to a 60 ml muscle bath. H represents histamine 0.2 μg ; PBZ represents Pyribenzamine (tripeleannamine) 5 μg ; Da represents dialyze of *Doriopsilla albopunctata* digestive glands equivalent to 0.4 g fresh tissue; W represents washed.

Thus, both chemical and pharmacological methods indicate the presence of histamine or histamine-like substances in the digestive gland of *Doriopsilla albopunctata*. If histamine is present even in a concentration of approximately 1 mg/g fresh tissue, can this account for the lethal effects of these dialyzates? Dialyzates or supernatant solutions of the digestive gland are lethal in mice at a dose level of 25 g/kg fresh gland. This represents about 25 mg/kg histamine or histamine-like substance. According to Spector (1955) the LD_{50} of histamine by intraperitoneal injection into mice is 12,930 mg/kg. We found in a few trial experiments that a dose of 100 mg/kg histamine intraperitoneally in mice was without effect. Thus, although histamine is present in the extracts of the digestive glands, the lethal effects of these extracts must be attributed to other substances.

Partial purification of the toxic substance

Three fractions of the digestive glands of specimens of *Anisodoris nobilis* from column chromatography (designated #1, #2, and #3) each equal in volume to the void volume, were collected. Only fraction #2 was active. Doses equivalent to 10 to 20 g/kg fresh digestive gland were lethal. Sublethal doses produced a characteristic sequence of changes in the behavior of the mice. They became lethargic and semi-comatose. They could be readily aroused by pinching the tail or by prodding. The muscles of the hind legs appeared to be affected in such a way that the animals walked or ran with an abnormal, slow gait. With large doses they would lie supine in the hand until disturbed. Respiration was slow and the body temperature was reduced. Further experiments (see below) established that these fractions produced severe and prolonged bradycardia and hypotension. The agent responsible, which is now being purified and identified, will be referred to here as dorid toxin.

Effects of dorid toxin in anesthetized rats

Aliquots of fraction #2 were injected intravenously into anesthetized rats (five experiments) in which the arterial pressure was recorded in the conventional

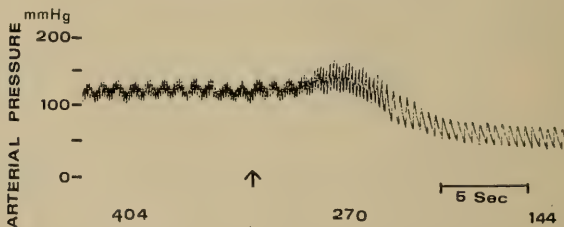


FIGURE 2. Arterial pressure of anesthetized rat. At arrow 0.075 ml of Fraction #2 from column chromatography of digestive glands of *Anisodoris nobilis* injected intravenously in 590 g rat. Figures indicate heart rate per min.

way from a cannula in one carotid artery. Electrocardiograms were recorded simultaneously from Lead II. A dose of 100 mg dry substance per kg body weight was lethal in 20 min. Heart rate fell from 390 to 150 per min and systolic pressure from 120 to 35 mm Hg within a few seconds of the injection. In four other animals doses of 14 to 22 mg/kg decreased the heart rate by 72% and the systolic arterial pressure by 53% within 30 seconds of the injection. A representative experiment is shown in Figure 2. The hypotension and bradycardia are remarkable for their long duration. Although no attempt was made to determine how long these effects lasted, they were still present for up to 60 min or when the experiments were terminated.

The hypotension occurs without decrease in pulse pressure, suggesting that peripheral vasodilation is not involved in its production. The hypertension produced by epinephrine (1 μ g) and norepinephrine (1 μ g) were approximately the same after administration of the toxin as before, indicating that the arteriolar musculature responds to sympathomimetic substances and is not paralyzed by dorid toxin. Carotid sinus reflexes, activated by clamping the remaining patent artery, were still present during the profound hypotension produced by dorid toxin. This suggests that the substance does not block ganglia, since this reflex arc is functional.

Effects of dorid toxin on isolated heart

Dorid toxin produced ventricular standstill in the perfused isolated frog heart. It blocked contraction of the hearts of specimens of *Mytilus californianus* and *Mercenaria mercenaria* *in vitro*, as well as that of the isolated spontaneously beating guinea pig atria. The effect on the latter heart preparation is shown in Figure 3. Dorid toxin has both negative chronotropic and inotropic effects in this preparation. Fifty per cent decrease in amplitude is produced by a concentration of about 20 μ g/ml of the eluates from Biogel P-2 columns of the digestive glands of specimens of *Anisodoris nobilis*.

Chemical properties of the dorid toxin

Since the dorid toxin was dialyzable through Spectrapor #1 dialysis tubing, the molecular weight must be under 6000 to 8000, the approximate cutoff for this tubing. Dorid toxin was not destroyed by heating in a boiling water bath for

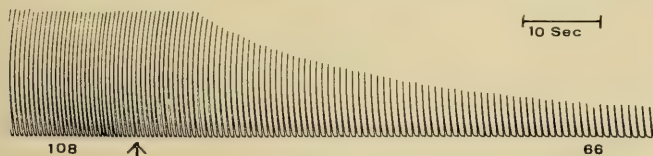


FIGURE 3. Effect of extract of *Anisodoris nobilis* on spontaneously beating guinea pig atria. Extract equivalent to 140 mg fresh tissue added at arrow to an 8 ml tissue bath. Figures indicate atrial rate per min.

20 to 25 min. To determine whether the dorid toxin is a protein, samples buffered with Tris at pH 8 were incubated with trypsin and chymotrypsin, the activities of which were confirmed using casein as a substrate. After incubation at 30° C for 20 hr, the solution was tested by intravenous injection into anesthetized rats and the arterial pressure and heart rate measured. The effects were the same as those obtained with comparable doses of untreated dorid toxin. Incubation with Pronase and assay on the isolated guinea pig atria indicated that no inactivation occurred. Therefore it is extremely unlikely that dorid toxin is a protein or a polypeptide.

DISCUSSION

Marine invertebrates contain a wide variety of active substances that affect the heart and peripheral vasculature. Generally, these substances have positive rather than negative inotropic and chronotropic effects on isolated hearts (Agarwal, Ligon and Greenberg, 1973; Kaul, Kulkarni, Weinheimer, Schmitz and Karns, 1977). The effects of dorid toxin are thus somewhat unusual. It is clearly not histamine, although histamine is present in crude extracts of the digestive gland. As discussed above, the amount of histamine present is far too low to account for the lethal effects, and the prolonged action of dorid toxin is not similar to that of histamine.

The posterior salivary glands of one genus of cephalopod (*Eledone*) contain a peptide that produces transient hypotension in mammals by peripheral vasodilation (Erspamer and Anastasi, 1962). Similar hypotensive peptides occur elsewhere (Erodös, Back and Sicuteri, 1966). Dorid toxin differs from these in having a prolonged duration of action—a difference of perhaps 100-fold or more.

The hypobranchial glands of gastropods of the families Muricidae and Thaididae contain choline esters such as murexine, (urocanylcholine), N-methyl-murexine, seneciylcholine, dihydromurexine, and acrylylcholine (cf. Whittaker, 1973; Bender, DeRiemer, Roberts, Rushton, Boothe, Mosher and Fuhrman, 1974). Murexine also occurs in the digestive glands of *Aplysia* (Blankenship, Langlois and Kittredge, 1975). We have been unable to demonstrate the presence of any cholinergic activity in the dialysates of dorid digestive glands either on the guinea pig ileum or the frog rectus abdominis muscle.

Surugutoxin is a ganglionic blocking agent from the Japanese ivory mollusk (*Babylonia japonica*) with a proven structure that combines 6-bromoindole, pteridine and myoinositol (Hayashi and Yamada, 1975). Although it produces moderate hypotension in mammals, the marked and prolonged bradycardia produced by dorid toxin differentiates the two substances. Other hypotensive substances that are as yet unidentified have been reported to be present in tectobranchs (Watson, 1973; Watson and Raynor, 1973; Carlos de Freitas, 1977) and in several other marine invertebrates (Kaul *et al.*, 1977). The relationship of dorid toxin to these unidentified compounds remains to be established.

Our interest in naturally occurring substances with pronounced pharmacological activity such as dorid toxin is in their usefulness as tools in research or as models for new drugs. Other investigators may be interested in the possibility that such toxins may be related to the defense of nudibranchs against their natural

predators. They have few predators and are reported to be rejected by fish (Garstang, 1890; Herdman, 1890; Thompson, 1960; Russell, 1966). Whether this is because of coloration, calcareous spicules, or noxious secretions is by no means clear. Perhaps toxic or noxious substances other than dorid toxin are produced by these animals. Certain of them (*e.g.*, *Hypsclodoris*, *Anisodoris*) do have pronounced odors. Dorid toxin may prove useful in both ecological studies and in pharmacology.

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SUMMARY

1. Aqueous extracts of digestive glands of specimens of the dorid nudibranchs *Cadlina flavomaculata*, *Doriopsilla albopunctata*, *Anisodoris nobilis*, *Archidoris montereyensis*, and *A. odhneri* were lethal when injected into shore crabs and when injected intraperitoneally into mice.

2. Aqueous extracts of the digestive glands of *Doriopsilla albopunctata* and of *Anisodoris nobilis* were shown by bioassay (guinea pig ileum) and by chemical determination to contain histamine. The amount present was far too small to account for the toxicity of the glands.

3. Extracts of the digestive glands of *Anisodoris nobilis* were fractionated by column chromatography on Biogel P-2 to yield an active fraction designated "dorid toxin." This produces lethargy and bradycardia in mice. In anesthetized rats it produces sustained (60 min or more) bradycardia and hypotension. On isolated hearts, especially spontaneously beating guinea pig atria, it has negative inotropic and chronotropic effects.

4. Dorid toxin has a molecular weight under 8000. It is heat stable and is not destroyed by trypsin, chymotrypsin or Pronase. It is therefore unlikely that it is a polypeptide.

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GUT STRUCTURE, FEEDING AND DIGESTION IN THE BRANCHIO-
BDELLID OLIGOCHAETE *CAMBARINCOLA MACRODONTA*
ELLIS 1912, AN ECTOSYMBIOTE OF THE FRESHWATER
CRAYFISH *PROCAMBARUS CLARKII*

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Members of the oligochaete family Branchiobdellidae live ectosymbiotically on freshwater astacid crustaceans and have been reported from North and Central America, Europe and most of Asia apart from the Indian sub-continent (Holt, 1960, 1973; Pop, 1965). Their morphology, anatomy and taxonomy are well documented (Moore, 1895; Yamaguchi, 1934; Clark, 1969 and references therein) and there have been many studies aimed at establishing the type of relationship which exists between the branchiobdellids and their hosts. Holt (1973) has reviewed these and summarizes the symbiotic status of those species which live on the surface of their hosts, outside the branchial chamber, as epizoic, since they graze on unicellular algae, bacterial gloea and associated microfauna. Species living within the branchial chamber though, are regarded by Holt as parasitic, since host tissues have often been reported in their gut contents.

This apparent dichotomy in feeding habits and, therefore, symbiotic status, in relation to the branchiobdellids' preferred locations on their hosts, was seen particularly clearly in a study by Grubda and Wiezbecka (1969), who injected radioactive chromium into the common host of *Branchiobdella hexadonta* and *B. pentadonta*. The former species, in the branchial chamber, was found to have taken up the radioactive label while the latter, living on the body surface outside the chamber, had not. Bishop (1968), however, using serological methods with *Cambarincola chiroccphala*, which is primarily a body surface dweller, found evidence of ingested host tissues in juveniles but not in adults, implying a difference in diet at different stages in the life history.

Apart from these observations on diet there is virtually no information available on the general nutritional physiology of branchiobdellids. Moore (1895) and Richards (1971) have provided some information on the anatomy and histology of the alimentary canal but nothing is known of the functions of its various regions in relation to digestion and absorption, of its ultrastructure, or of the types of enzymes and other secretions produced within it. Accordingly, a study has been undertaken of these and related aspects of the nutrition of *Cambarincola macrodonta*, a branchiobdellid living on the rostrum and carapace of the North American crayfish *Procambarus clarkii*.

MATERIALS AND METHODS

Cambarincola macrodonta Ellis 1912 was obtained during July and August 1974 from specimens of *Procambarus clarkii* collected in California by a com-

mercial supplier and flown to the Marine Biological Laboratory, Woods Hole, for use in neurophysiological research. The branchiobdellids survived well after removal from their hosts; they were kept for up to ten days in shallow dishes of well-aerated fresh water and fed daily on chopped fish liver and clotted fish blood.

The structure of the feeding apparatus and alimentary system, the sites of production and action of the various enzymic and other secretions concerned in feeding and digestion, and the sequence of events during digestion and absorption were studied in starved and blood- or liver-fed individuals using the histological and histochemical methods described in our earlier account of similar studies on the symbiotic polychaete *Histriobdella homari* (Jennings and Gelder, 1976). The histochemical methods used included the indoxyl acetate method for non-specific esterase (Holt, 1958), the L-leucyl β -naphthylamide hydrochloride method for arylamidases (Burstone and Folk, 1956), the naphthyl AS-BI phosphate method for acid and alkaline phosphatase (Burstone, 1958) and the post-coupling 6-bromo-2-naphthyl β -D-glucopyruronoside method for β -glucuronidase (Pearse, 1972). Esterases demonstrated by the Holt method were further characterized using procedures and interpretations given by Pearse (1972) and Hassall and Jennings (1975).

For ultrastructural studies intact branchiobdellids, and others cut into two or more pieces, were fixed for 1 hr at 4° C in 4% glutaraldehyde in 0.1 M phosphate buffer, rinsed in buffer and post-fixed for 1 hr in 2% osmium tetroxide in phosphate buffer (after Bondi and Facchini, 1972). Dehydration in graded ethanols was followed by embedding in epon; sections were then prepared and stained by standard methods for examination with the optical and electron microscopes.

The nature of the normal diet was determined from direct and histological examination of the gut contents of specimens newly removed from their hosts; this was supplemented by similar examination of the host's body surface in the areas favored by the branchiobdellids. The feeding mechanism was studied by direct observation, using bright field or dark ground illumination, of individuals starved for 2-3 days and then presented with either the natural food or pieces of fresh or boiled fish liver; the roles of the various muscles involved were deduced from these observations and from their distribution within the anterior portion of the body as revealed in histological and ultrastructural preparations.

OBSERVATIONS AND RESULTS

Cambarincola macrodonta when mature is 2.0-2.5 mm long and bears a well-defined posterior sucker which is used for adhesion to its host. The body, composed of fifteen segments, is held at varying angles to the host's body surface and is often in motion, making gentle swaying or sweeping movements apparently in search of food. Favored points of attachment to the host are the rostrum and dorsal surface of the cephalothorax, but individuals were occasionally found elsewhere on the body surface.

Structure of the alimentary canal

The alimentary canal in *C. macrodonta* (Figs. 1A, 1B and 2) consists of an oral region, bounded by dorsal and ventral lips derived from the peristomium and

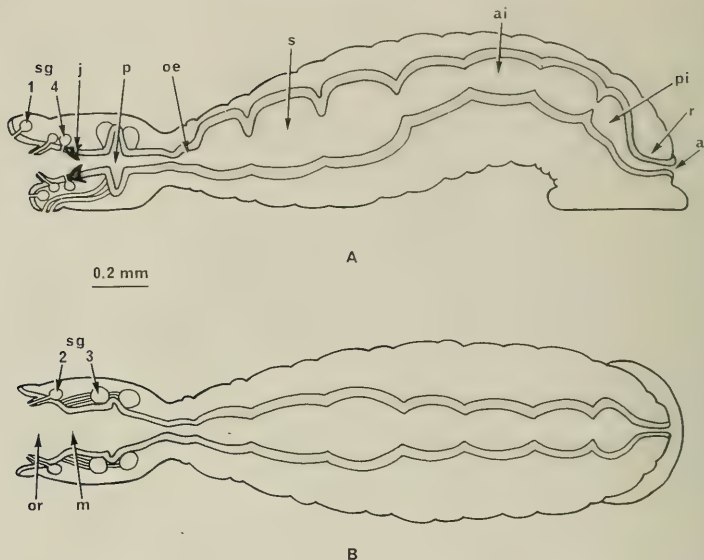


FIGURE 1. *Cambarincola macrodonta*. Schematic longitudinal sections (A, sagittal and B, horizontal) through a mature individual to show the alimentary canal and associated structures. Abbreviations are: a, anus; ai, three-chambered anterior intestine; j, jaws; m, mouth; oe, oesophagus; or, oral region; p, pharynx; pi, posterior intestine; r, rectum; s, three-chambered stomach; sg 1-4, salivary glands types 1, 2, 3 and 4. The scale bar indicates the overall dimensions; the gut wall is shown thicker than in life.

sometimes described as an oral sucker, the mouth, pharynx, oesophagus, three-chambered stomach, three-chambered anterior intestine, a posterior intestine, rectum and anus. Four groups of salivary glands open into the oral region or onto its ventral surface and a pair of sclerotized jaws are located on the dorsal and ventral walls of the pharynx. The stomach, anterior intestine and the first third of the posterior intestine are surrounded by the gastric vascular sinus of the blood system.

The oral region, mouth, pharynx, jaws and salivary glands. This anterior portion of the gut lies within the first four segments, which form a distinct "head" region. It is lined throughout by cuticle approximately $1\ \mu\text{m}$ thick and continuous with the cuticle covering the general body surface. Ultrastructurally the cuticle consists of six layers of collagen fibers which are stacked at right angles to each other to form a lattice structure; this rests on epithelial cells from which microvilli extend to penetrate the lattice. The remaining spaces within the lattice are filled with deeply staining granules and filaments. Externally the cuticle is bounded by a thin, densely staining layer which bears short fusiform bodies (Figs. 3, 4, 5) connected to each other by reticulated filaments.

The oral region is bounded by a large dorsal lip and a slightly smaller ventral one; both lips bear a median fissure and their cuticular covering is penetrated at intervals by bundles of sensory cilia (Fig. 3) which may project from the cuticle

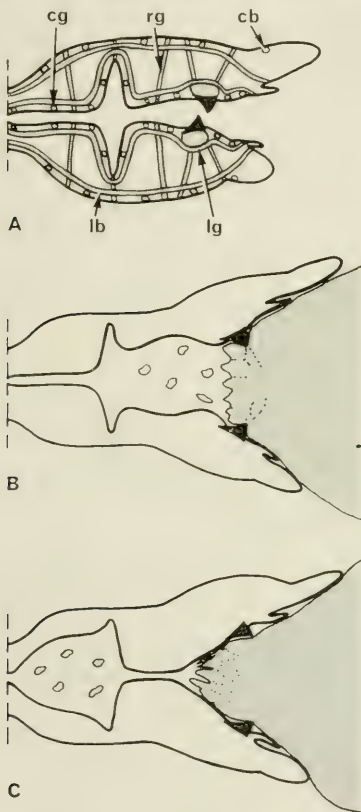


FIGURE 2. *C. macrodonta*. Schematic longitudinal sections of the anterior region showing stages in the feeding process. A. The resting stage, showing also the muscles involved in ingestion. B. The oral region is distended and applied to a food mass (shaded); the jaws are pressing on the food and detaching fragments from its surface which are then sucked into the anterior pharynx. C. The oral region and, especially, the anterior pharynx are contracting and the detached food has passed into the posterior pharynx from which it will move onwards into the oesophagus. Abbreviations are: cb, circular muscles of the body wall; cg, circular muscles of the anterior gut; lb, longitudinal muscles of the body wall; lg, longitudinal muscles of the anterior gut; rg, radial muscles of the anterior gut.

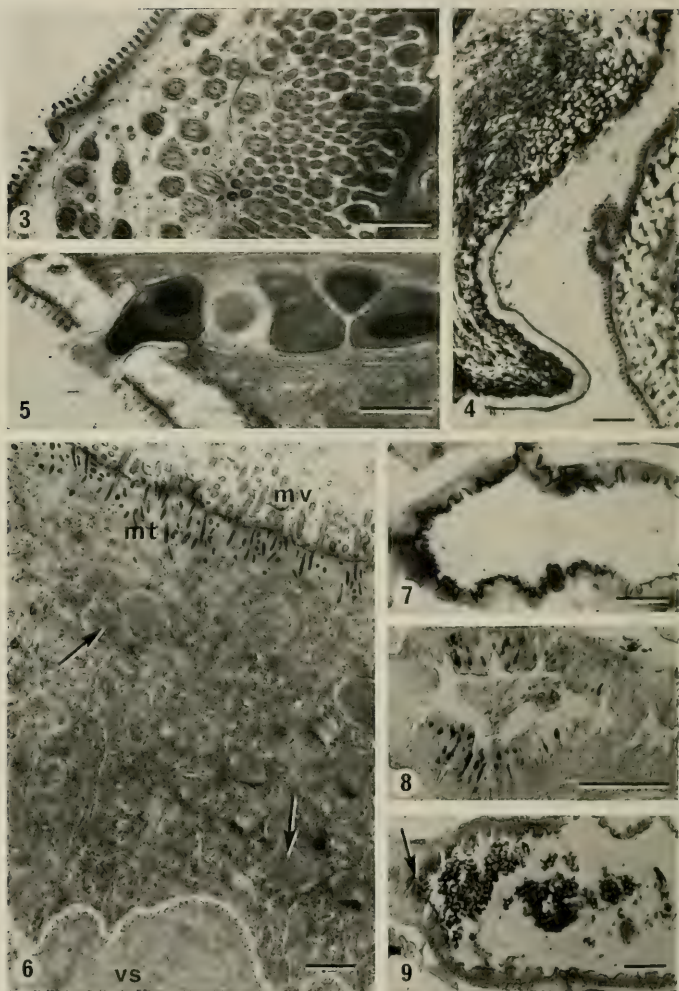


FIGURE 3. *C. macrodonta*. An electron micrograph of a section through a sensory ciliary bundle in the oral region. Fixation was in glutaraldehyde and osmium tetroxide (following Bondi and Facchini, 1972); the section was stained with uranyl acetate and lead citrate. The scale bar represents 1 μ m.

up to a height of 8 μ m. Each cilium is surrounded by microvilli and the cuticle immediately around each bundle increases in thickness, apparently for support.

The mouth opens directly into the pharynx, without the intervention of a buccal cavity; it is surrounded by eight papillae through which one group of salivary glands discharge (Figs. 1A and 1B). The jaws lie immediately behind the mouth, one dorsally and the other ventrally on the pharyngeal wall. Each jaw is denticulate and roughly triangular; the dorsal jaw bears five denticles and the ventral four, arranged so that they interdigitate when the jaws meet. The jaws are formed from thickened folds of the pharyngeal cuticle (Fig. 4); their basal regions consist of unmodified cuticle but the denticles are composed of folded collagen fibers lying in a deeply-staining matrix. Each denticle is covered by a lightly-staining homogeneous layer, approximately 0.3 μ m thick, which is penetrated by atrophied microvilli. The fusiform bodies which are a characteristic feature of the cuticle surface elsewhere are much abraded on the distal portions of the denticles, but remnants can be found near their bases and intact ones elsewhere on the jaws.

Halfway along its length the pharynx expands into two diverticula-like folds, one dorsally and the other ventrally (Fig. 1A). It then continues posteriorly to join the oesophagus in the first body segment.

The entire alimentary canal has a well-developed musculature, lying below the epithelial layer lining the lumen, and consisting of an inner layer of circularly

FIGURE 4. *C. macrodonta*. An electron micrograph of a sagittal section through a denticle (left) of the dorsal jaw, showing the thickening and modification of the cuticle as compared with the normal cuticle (right) of the pharynx. Preparation and staining of the section were the same as for Figure 3; the scale bar represents 1 μ m.

FIGURE 5. *C. macrodonta*. An electron micrograph of a section through the duct of a cell from a salivary gland type 2, showing secretion bodies passing onto the lower lip. Preparation and staining of the section were the same as for Figure 3; the scale bar represents 1 μ m.

FIGURE 6. *C. macrodonta*. An electron micrograph of a section through the stomach wall, showing part of an epithelial cell with microvilli (mv) below which can be seen microtubules (mt) derived from the rough endoplasmic reticulum and containing densely staining secretion. The cytoplasm contains lipid droplets (arrowed) and folded intrusions of the basal surface; the lumina of the intrusions are continuous with the vascular sinus, part of which (vs) can be seen below the cell. Preparation and staining of the section were the same as for Figure 3; the scale bar represents 1 μ m.

FIGURE 7. *C. macrodonta*. A photomicrograph of a longitudinal section through the anterior intestine in segment 7 of a specimen fixed three hours after feeding on the natural food. The distal regions of the epithelial cells show intense alkaline phosphatase activity (black). Fixation was in 10% formalin; the section was treated by the azo-dye method for alkaline phosphatase (Burstone, 1958). The scale bar represents 20 μ m.

FIGURE 8. *C. macrodonta*. A photomicrograph of a longitudinal section through the anterior intestine in segment 7 of a specimen fixed fifteen minutes after feeding on the natural food. The gland cells show strong acid phosphatase activity (black). Fixation was in 10% formalin; the section was treated by the azo-dye method for acid phosphatase (Burstone, 1958). The scale bar represents 20 μ m.

FIGURE 9. *C. macrodonta*. A photomicrograph of a longitudinal section through the anterior intestine in segment 7 of a specimen fixed three hours after feeding on the natural food and showing strong extracellular arylamidase activity (black) in food particles lying in the intestinal lumen. Less intense activity can be seen in the distal regions of some of the epithelial cells (arrowed). Fixation was in 10% formalin; the section was treated by the Burstone and Folk (1956) method for arylamidases. The scale bar represents 20 μ m.

oriented fibers and an outer layer of longitudinal ones. In the pharynx (Fig. 2A) the circular fibers are organized into distinct bands which lie between the lower portions of the subcuticular cells; the band passing below the jaws is considerably thickened and the bases and lateral margins of the jaws are bound to the muscles by fibrous connections. The longitudinal muscles are attached anteriorly to the pharyngeal cuticle by similar connections in the region of the oral papillae; they run posteriorly over the circular bands and continue down the length of the gut. The circular and longitudinally oriented musculature of the pharynx is supplemented by a set of radial muscles which run from the pharyngeal cuticle, between the cells of the subcuticular epithelium, to their attachment to the cuticle of the body wall. In the jaw region the radial muscles are attached to the bases of the jaws, along with the circular muscle band in that area, and in the region of the dorsal and ventral diverticular folds they are attached to the distal regions of the folds so that their contraction or relaxation alters the shape of each fold.

The body wall is provided with a similar musculature to the gut, but consists of outer circular and inner longitudinal layers (Fig. 2A). Anteriorly, the longitudinal layers slant inward from their usual position below the dermis and become attached to the cuticle lining the oral region, near the oral papillae. Both layers of the body wall musculature, in the "head" region, supplement the pharyngeal musculature during ingestion of the food.

The four groups of salivary glands, hereafter designated types 1, 2, 3 and 4, each consist of paired pyriform cells which discharge their secretions through narrow ducts opening at various points on the lips or within the oral region (Figs. 1A, and 1B). The secretions are non-enzymic and vary somewhat in their staining reactions, implying different roles in the feeding process. Salivary glands type 1 consist of six pairs of gland cells, three pairs being located in the dorsal lip and three in the ventral, which discharge onto the oral surfaces of the lips a viscous secretion staining strongly with aniline blue and Best's carmine.

The four pairs of salivary gland cells type 2 are associated with the oral papillae. Their secretions, unlike those of the other three types, stain with Alcian blue and the periodic acid-Schiff reaction which indicates an acid mucopolysaccharide complex. The ducts of the cells traverse the papillae to open at the tips so that the extruded mucoid secretions spread over the entire surface of each papilla.

Salivary glands type 3 consist of four pairs of cells situated laterally around the dorsal diverticulum of the pharynx. Their ducts run ventrally beneath the mouth and divide before opening onto the ventral surface of the "head" below the oral region. The secretions stain lightly but in different shades with aniline blue; ultrastructurally each secretion body consists of a homogeneous cortex surrounding a core of deeper-staining microspheres, and as they are discharged the bodies hydrate and fuse to form a fibrous mucoid substance (Fig. 5). The gland cells and secretions have the same morphology and staining properties as those found in the segmental and acetabular adhesive glands and the secretions, therefore, are believed to be adhesive in function.

The two pairs of salivary gland cells type 4 are located dorsally and ventrally immediately in front of the jaws. They discharge through fine pores in the

pharyngeal cuticle near the jaws; the secretions stain strongly with acid fuchsin but their function is not known.

The oesophagus, stomach, intestine, rectum and anus. The oesophagus is a short ciliated tube running from its junction with the pharynx in the first body segment (segment five of the entire body) to its opening into the stomach in body segment two. The stomach (Figs. 1A and 1B) extends to the fourth body segment; the intersegmental septa cause constrictions which partially divide it into three chambers but the divisions have no functional significance. The stomach wall consists of a ciliated epithelium, one cell thick, which is underlain by the circular and longitudinal layers of the gut musculature. Between the epithelium and muscular layers, however, there is the gastric vascular sinus; this arises from the dorsal longitudinal blood vessel which in the third body segment becomes flattened against the stomach and divides repeatedly into a plexus of smaller vessels. These then merge into a single sinus which completely surrounds the epithelial layer. The basal surfaces of the epithelial cells form intrusions running deep within the cells, and the vascular sinus is continuous with these so that the vascular fluid is in intimate contact with most regions of the cells (Fig. 6). The vascular sinus continues in this form down the length of the gut until after the commencement of the posterior intestine; it then reforms into a plexus of vessels which eventually unite to form the posterior part of the dorsal longitudinal vessel.

The gastric epithelial cells (Fig. 6) are all of one columnar type, 10 to 14 μm in height and uniformly ciliated with unbranched microvilli 1.5 μm tall. In the terminal web layer immediately below the microvilli the rough endoplasmic reticulum, present in normal form throughout most of the cell, here loses its ribosomes and the lumen increases in diameter from 45 to 180 nm. The dilated organelles become microtubules packed with densely-staining material, extremely prominent immediately below the microvillar layer (Fig. 6), and this is eventually passed out into the gut lumen. The nucleus of the cell lies centrally and the surrounding cytoplasm contains rough endoplasmic reticulum, mitochondria, lipid droplets and the intrusions of basal cell wall containing vascular fluid.

In histochemical preparations the cytoplasm in starved individuals shows weak reactions for A-esterases, acid phosphatase and arylamidases; the demonstrable amounts of the latter two enzymes, however, increase when food enters the stomach.

The link between the stomach and the anterior intestine is marked by a well-defined constriction as the gut passes through the septum between body segments four and five. The anterior intestine occupies segments five, six, and seven and the intersegmental septa, as with the stomach, give it a three-chambered appearance but, again, no functional significance for this division was found. There is, however, a difference in the cellular structure of the epithelial layer; that in segments five and six is virtually identical with that of the stomach but in segment seven the ciliated cells are interspersed with conical, unciliated gland cells characterized by the presence of Golgi apparatus and secretory granules. The distal regions of both types of cell show intense positive reactions for alkaline phosphatase, in both starved and fed individuals (Fig. 7), but the gland cells also show a strong

reaction, throughout their cytoplasm, for acid phosphatase (Fig. 8) and arylamidases. In histological preparations the gland cells are further differentiated from the others by their affinity for acid fuchsin.

The junction between the anterior and posterior intestines is marked by a prominent constriction where the gut passes through the septum separating body segments seven and eight. The posterior intestine occupies segments eight and nine and has much the same structure as the anterior intestine except that gland cells are absent and the epithelial cells are smaller being only 5 to 8 μ m tall, and cuboidal rather than columnar. More significant differences, though, are their lack of the layer of secretion-packed microtubules below the microvillar surface, and their lack of the enzymes so readily demonstrable in the more anterior regions of the gut; tests for esterases, arylamidases and phosphatases consistently gave negative results irrespective of the animal's nutritive state.

The rectum occupies body segment ten; its epithelium lacks cilia apart from a small area of the ventral wall where cilia are retained, apparently to keep the rectal contents in motion. The cells bear a cuticle, similar to that of the epidermis, oral region, and pharynx, and they terminate at the anus, which opens to the exterior on the dorsal surface of the tenth body segment (segment fourteen of the entire body, if the head is included). The circular muscle band around the anal opening is thickened to form a distinct sphincter.

The food and feeding mechanism

Cambarincola macrodonta feeds on the epizoid microflora and fauna present on its host's body surface. Examination of the gut contents of individuals newly removed from infested crayfish revealed diatoms, bacteria, cells of filamentous algae, protozoa (especially stalked and free-swimming ciliates), rotifers and, occasionally, larval and adult copepods. The gut also often contained fibrous or granular proteinaceous materials; these could not be positively identified but their appearance and staining reactions strongly suggested that they were derived from the host's food, which in the laboratory consisted of small pieces of fish and fish offal. This interpretation was supported by the readiness with which, *in vitro*, the branchiobdellids ingested suitably sized particles of these foods and of clotted blood or liver.

There was no evidence of ingestion of host tissues, nor of abrasion or other damage to the host's body surface ascribable to the branchiobdellid.

The feeding process varies slightly, depending upon whether epizoid organisms are being detached from the host's cuticle, or whether particles are being removed from a larger mass of soft food, but the mode of operation of the jaws and pharynx is essentially the same in both cases.

The shape of the pharynx and the relative positions of the lips and jaws in the resting individual are shown in Fig. 2A. When feeding begins the "head" is brought up to the food and the lips are pulled forwards and outwards, first by slight contraction of the subepidermal circular muscles which increases pressure within the head by acting on fluid-filled spaces, and then by greater contraction of the longitudinal muscles. The opened lips are closely applied to the food-bearing substratum, or a food mass, by movement of the entire head. The circular muscles

around the mouth then relax while the radial muscles of this region, and especially those attached to the jaws, contract. This opens the mouth, presses it to the food, and rotates the jaws so that they point anteriorly rather than dorsally and ventrally (Fig. 2B). Contraction of the radial muscles attached to the anterior pharynx causes it to dilate and thereby create a suction which draws the food through the mouth; at the same time strong contractions of the circular muscles of the oral region and lips, and appropriate relaxation of the longitudinal muscles, cause the jaws to swing inwards against the food. When the latter is a soft mass, such as the liver used experimentally, this section of the jaws either detaches fragments which are sucked onwards into the dilated anterior pharynx or else sets up lines of stress along which the food eventually fractures due to the pharyngeal suction. The grip on a food mass by the jaws is often followed by rapid relaxation of the lips and then a further stretching and contraction of these structures over more of the food which is thus squeezed further into the mouth. This process continues, with the jaws acting both as a ratchet mechanism facilitating passage of food through the mouth and as a part of the detaching and fragmenting process, until ingestion is completed.

When the food mass is relatively small, as when the lips are applied to a group of rotifers or stalked ciliates, the pharyngeal suction draws the organisms through the mouth and into a position where the jaws, swinging inwards, can meet across them, the denticles interdigitate and the organisms are clipped off and sucked into the pharynx.

Specimens placed on the host's gills were occasionally seen to feed by drawing a single gill filament through the mouth until the tip was within the pharynx. The branchiobdellid then drew its head away from the filament so that the jaws scraped off encrusting microorganisms. Subsequent examination showed the surface of the filament to be undamaged.

The final stage in feeding, the actual ingestion of detached fragments, is achieved by contraction of the anterior pharynx and dilation of the posterior region. This creates a peristaltic wave which urges the food through the pharynx to the oesophagus (Fig. 2C). From here ciliary action conveys it onwards into the stomach.

During feeding the salivary secretions are discharged onto the food and are ingested with it, but their precise role is unknown. They presumably help the lips to secure a grip on a food mass or the substratum from which microorganisms are to be removed, and those from type 2 glands may act as a lubricant or transport medium. Food particles are not bound together by the secretions though, and their non-enzymic nature precludes a digestive function.

Digestion

Food entering the stomach from the oesophagus usually stains to some degree with Alcian blue and periodic acid-Schiff; partly because of its intrinsic affinity for these stains in the case of the natural food but also due to admixture with salivary secretions during ingestion. It is held in the stomach for varying lengths of time, from fifteen minutes up to a maximum of four hours; during its stay material such as experimentally-fed liver is disrupted and the cellular contents of

natural food organisms lose their identity and become homogeneous. The food shows increasingly strong reactions for arylamidase and acid phosphatase and the enzymes responsible obviously originate in the gastric epithelium as there is a concomitant increase there in the demonstrable amounts of these enzymes. The histochemical reactions are strongest in the extreme distal region which contains the swollen and electron-dense microtubules (Fig. 6). It seems likely, therefore, that the enzymes are produced by these organelles. Discharge of membrane-bound materials from the epithelium was never observed however, and the precise method of secretion of the enzymes remains unknown.

The level of A-esterases present in the gastric epithelium, in contrast, remains the same irrespective of the amount or condition of the stomach contents; there was no evidence suggesting secretion of A-esterases into the lumen and such enzymes seem to play no part in digestion.

No histochemical or ultrastructural evidence was apparent to suggest absorption by the gastric epithelium.

The digesting food is moved around within the stomach by ciliary action and general body movements, and is eventually passed into the anterior intestine. Here the gland cells in the epithelium, which at all times show strong reactions for acid phosphatase (Fig. 8) and arylamidase, discharge their secretions and there is a dramatic increase in the level of these enzymes in the digesting food (Fig. 9). The food becomes quite homogeneous, apart from indigestible elements such as diatom cases and copepod exoskeletons, loses its affinity for Alcian blue and periodic acid-Schiff, and its volume is progressively reduced as soluble components are absorbed into the intestinal epithelium. The alkaline phosphatase consistently present in the distal region of the epithelium (Fig. 7) is probably concerned in this process. As materials enter the epithelial cells the cells develop arylamidase activity and this eventually becomes as intense as that seen in the gut lumen. It then fades, as intracellular digestion is completed and the products pass into the gastric vascular sinus for distribution around the body.

Indigestible residues are moved by ciliary action into the posterior intestine, where they accumulate, and then eventually into the cuticle-lined rectum from which they are voided via the anus.

Tests for β -glucuronidase activity in the gut epithelium were consistently negative; the enzyme was occasionally demonstrated in the gut contents of naturally-fed individuals but this was confined to clumps of ingested microorganisms and was clearly produced by them and not by the branchiobdellid.

Food reserves

Lipid droplets occur in the gastric and intestinal epithelium but the principal site of lipid storage is in the chloragocytes which surround the stomach and anterior intestine. No significant reserves of glycogen were found in any part of the body.

DISCUSSION

The pattern of nutritional physiology emerging from these observations on *Cambarincola macrodonta* is of interest with regard to both the symbiotic status

of this type of branchiobdellid and the general relationships of the Branchiobdellidae to other groups of annelids, especially the Hirudinea.

C. macrodonta would seem to be, primarily, an epizoic grazer with a life style of the type recognized by Holt (1973) as characteristic of most branchiobdellids. It is also, very probably, an opportunistic commensal capable of capturing and ingesting particles of the host's food when these are released from a larger mass by the shredding action of the crayfish's feeding mechanism. The readiness with which fragments of animal tissues are ingested *in vitro*, which supports this conclusion, has also been observed in a second species, *C. chirocephala*, epizoic on the crayfish *Orconectes orconectes longidigitus*, (Gelder, 1978) and it is possible, therefore, that opportunistic commensalism may be a normal supplement to microfloral and -faunal grazing in most branchiobdellids which live outside their host's branchial chamber. *C. chirocephala* was occasionally also found on its host's gills so that gill-dwelling species, too, may have this supplemental form of nutrition. An epizoic gill-dweller from another phylum, the triclad turbellarian *Bdelloura candida*, which lives mainly on the gill-books of the horse-shoe crab *Limulus polyphemus* appears, in fact, to depend almost entirely on this type of commensalism; the triclad feeds on fragments of the host's food which become detached during feeding and are then swept beneath the host and around the gill-books by the ventilating mechanism (Jennings, 1977; Lauer and Fried, 1977). In *Branchiobdella hexadonta*, though, from the gills of *Potamobius pallipes*, the guts of several specimens examined contained only epizoic microorganisms and nothing was found to suggest ingestion of either host tissues or the host's food (Gelder, 1978).

Further support for the idea that the diet in *C. macrodonta* normally includes fragments of the host's food (which is probably largely proteinaceous, in view of crayfishes' feeding habits) comes from the dominance of arylamidases (exopeptidases) in the digestive physiology. An unexpected feature of the latter, though, in the context of grazing ectosymbiosis, is the absence of endogenous β -glucuronidase. This enzyme is important in digestion in other microfloral grazers such as the symbiotic polychaete *Histriobdella homari* (Jennings and Gelder, 1976) and the free-living *Ctenodrilus serratus* (Gelder, 1978); its absence from *C. macrodonta* is inexplicable unless its role in digestion is performed either by the very small amount which enters the gut within ingested microorganisms, or by other enzymes as yet undetected.

With regard to the taxonomic relationships of the Branchiobdellidae, Holt (1965) suggests that they, the Acanthobdellidae and the Hirudinea are all clitellates which have evolved independently from oligochaete stock but which show convergence in their adaptations to ectosymbiotic life. The present observations support this view, insofar as feeding and digestion in the branchiobdellids and hirudineans are concerned; so little is known of acanthobdellid nutrition, though, that a comparison with them is impossible.

The feeding mechanism in the branchiobdellids consists of relatively simple modifications of the stomodaeal region; the jaws are formed from the thickened cuticular lining and the musculature which operates them is little modified from the basic oligochaete pattern. This contrasts sharply with the situation in the microfloral-grazing polychaete *Histriobdella homari* where the stomodaeal region is much

modified to form an exceedingly complicated grazing mechanism (Jennings and Gelder, 1976), and lends further support to the idea that the branchiobdellids are not exclusively grazers. The hirudinean jaws are similarly formed from simple thickening of the stomodaeal cuticle but their triradiate arrangement, as compared with the dorso-ventral pairing in the branchiobdellids, is sufficiently different to dispel the possibility of any close affinities between the two groups.

The salivary secretions in *C. macrodonta* differ from those of hirudineans in showing little detectable specialization in constitution and function; only a proportion are mucoid and none are enzymic. In sanguivorous leeches, in contrast, the specialized anticoagulant properties of the saliva are well known (see, for example, Haycraft, 1884, on *Hirudo medicinalis* and Stammers, 1950, on *Haemadipsa scylanica*) and a B-esterase, believed to have a digestive function, is present in the saliva of the fish leech *Piscicola geometra* (van der Lande, 1968).

There appears to have been more convergence, however, in the digestive physiology of the two groups. In the leeches, as in *C. macrodonta*, it is dominated by exopeptidases which are characterized histochemically as arylamidases; in both predatory and ectoparasitic species these enzymes are consistently present throughout the digestive epithelium and are responsible for extra- and intracellular proteolysis (Jennings and van der Lande, 1967; van der Lande, 1972). The same situation occurs in *C. macrodonta*, except that here emphasis on this type of protease is taken even further with differentiation of specific intestinal glands for their production. In the leeches this emphasis on proteolysis, albeit exopeptic, is understandable in relation to the diet, but this is not so in the branchiobdellid unless the opportunistic commensal component of feeding is of greater importance than the grazing one. A further possibility is that the latter provides a larger proportion of animal organisms high in protein content, such as protozoa and rotifers, than it does of filamentous algae and bacteria. In this connection, the deficiency in β -glucuronidase discussed earlier may be extremely significant. A major difference, though, in leech and branchiobdellid digestive processes is the duration of proteolysis; in *C. macrodonta* it occurs, as in most animals, in a matter of hours while in the leeches it can occupy days or even months. There is also strong evidence of bacterial involvement in leech digestion which might account for the absence of endopeptidases, but nothing was seen in the branchiobdellid to suggest a similar situation.

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SUMMARY

1. The branchiobdellid *Cambarincola macrodonta* is an ectosymbiote of the crayfish *Procambarus clarkii* and feeds by grazing on the microflora and fauna of the host's body surface. It is likely, however, that this type of feeding is supplemented by opportunistic commensalism in which particles of the host's food are captured and ingested.

2. The feeding mechanism is simple, there is little apparent specialization of function in the salivary glands and there are no evident adaptive features in either gut structure or function.

3. Digestion is both intra- and extracellular and is dominated by arylamidases (exopeptidases).

4. The nutritional physiology shows some similarities to that of the Hirudinea, notably in the emphasis on exopeptic digestion, but it is concluded that these reflect convergence rather than taxonomic affinity.

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THE EFFECT OF DIFFERENT ZOOXANTHELLAE ON THE GROWTH OF EXPERIMENTALLY REINFECTED HOSTS

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The dinoflagellate algae called zooxanthellae are fundamental in the economy of coral reefs. Many aspects of this widespread association have been reviewed (Droop, 1963; Muscatine, Pool and Trench, 1975; and Taylor, 1973a, b, 1974, 1975). Zooxanthellae may enable the symbiotic association to function as a primary producer (Franzisket, 1969, 1970; Johannes, 1974; Porter, 1976); contribute to the general nutrition of the host animal (Muscatine, 1971, 1973; Trench, 1971a, b, c, 1974; Muscatine and Porter, 1977); facilitate calcification in scleractinian corals (Goreau, 1961; Muscatine, 1971; Vandermeulen and Muscatine, 1974; Clausen and Roth, 1975); or function in obtaining or conserving scarce nutrients (D'Elia, 1977; D'Elia and Webb, 1977; Muscatine and D'Elia, 1978). These algae, shown to be *Gymnodinium*-like dinoflagellates by Kawaguti (1944) were first axenically cultured by McLaughlin and Zahl (1959, 1966). They are morphologically very similar across the range of host animals. Early light and electron microscopic studies gave little ground for considering that the symbionts found in different hosts were different (Freudenthal, 1962; Kevin, Hall, McLaughlin and Zahl, 1969). Freudenthal (1962) described the zooxanthellae from the scyphozoan *Cassiopea* sp. as *Symbiodinium microadriaticum*. Taylor (1971a) felt all gymnodinoid zooxanthellae might be but a single pandemic species, which he returned to the genus *Gymnodinium*. More recent workers use the original *Symbiodinium* (Schoenberg, 1976) and both names are found in the current literature. In addition to the nomenclatural differences at the generic level, the question of whether there is a single species of zooxanthella found in almost all marine benthic invertebrates as Taylor suggested (1974), or whether there may be several different zooxanthellae, still remains.

Genetic differences between the zooxanthellae could result from geographical, environmental or host specific factors. McLaughlin and Zahl (1966) suggested that it would be unwise to generalize on the similarity of different zooxanthellae from different habitats. Others (Jokiel and Coles, 1977; Dustan, 1975; and Kinzie, 1970), while giving no evidence from the algae themselves, have interpreted physiological differences between corals in different locations or habitats as suggesting the existence of different algal symbionts. Trench (1971b, c) showed that zooxanthellae from different hosts excrete different amounts of photosynthetic substances under the same conditions *in vitro*. Strains of zooxanthellae isolated from different hosts have been shown to exhibit different isozyme patterns (Schoenberg and Trench, 1976; Schoenberg, 1976). Schoenberg (1976) and Steele (1975) have recently suggested that some strains of zooxanthellae are in fact morphologically different.

While these studies support the idea that there are different kinds of zooxanthellae, the important question (ecologically though perhaps not taxonomically) is whether algal strain differences influence the host algal association. Advantages may accrue to either the alga or the host and need be neither reciprocal nor symmetrical. Differences in the ability of several strains of zooxanthellae to infect aposymbiotic hosts have been shown by Trench (1971c), Kinzie (1974), and Schoenberg (1976); suggesting that uninfected potential hosts have the ability to discriminate between different algae. Algal growth after reinfection varies. Homologous—"correct"—algae increase in numbers faster than heterologous—"foreign"—strains in these experimental infections (Schoenberg and Trench, 1976; Schoenberg, 1976).

Taken together the studies cited above show that different strains of the zooxanthella *Symbiodinium microadriaticum* exist, and that these strains differ in their ability to infect aposymbiotic coelenterate polyps. Further, the reinfection experiments suggest that those strains that can initiate a symbiotic association differ in their ability to increase in the host's tissues, and that the homologous strain shows the highest growth rate.

The advantages to the host infected with the normal symbiotic algae are well known. A number of workers have shown that in several algal invertebrate associations the algae contribute to the growth rate or maturation of the host (Muscatine, 1961; Muscatine and Lenhoff, 1965; Karakashian, 1963; Taylor, 1971b; Provasoli, Yamasu and Manton, 1968; Sugiura, 1964). Except for the work of Muscatine on *Anthopleura* and Sugiura on *Mastigias*, these studies have been conducted on systems other than the zooxanthella/coelenterate association.

While anemones of the genus *Aiptasia* are satisfactory test animals for many studies on zooxanthellae/coelenterate interactions, this system suffers from the fact that the polyps generally show neither sexual reproduction in the laboratory nor regularly consistent asexual budding, precluding the use of numerical increase as a measure of growth. Their great contractility and flexibility make linear measurements practically meaningless.

To overcome these problems in assessing the effect of different zooxanthellae on the metabolism of *Aiptasia*, we used the increase in the number of tentacles as a measure of the growth of the host. As will be shown later, the number of tentacles is correlated with the weight of the animal, yet data on tentacle number can be repeatedly obtained from an individual whereas dry weight cannot. This method is sensitive enough to enable us to differentiate between the effects of different zooxanthellae. Preliminary results have shown that this method is an accurate and practical measure of host growth (Kinzie, Araki and Kia, 1977). The experiments discussed here were designed to determine if zooxanthellae isolated from different hosts differ in their ability to contribute to the growth of experimentally reinfected *Aiptasia* polyps.

MATERIALS AND METHODS

Algae

Homologous algae were isolated from *Aiptasia pulchella* growing in the wet tables supplied with unfiltered running sea water at the Pacific Biomedical Re-

search Center in Honolulu, Hawaii. The tables were outside but shaded by a roof. Algae from several polyps were pooled to initiate the cultures. The other coelenterate host used as a source of zooxanthellae was the scyphozoan *Cassiopea xamachana*. Algae were isolated from three individuals obtained from a depth of about 2 m at the Discovery Bay Marine Laboratory in Jamaica. Two mollusk hosts were used as algal sources. One, *Tridacna maxima*, was obtained from a depth of 3 m near the Mid Pacific Marine Laboratory at Enewetak in the Marshall Islands. Algae were isolated from the mantle tissue of a single individual. Algae from the nudibranch *Melibe pilosa* were isolated from a single individual obtained from a depth of less than 1 m on the Ala Moana reef flat off Kewalo Basin, Oahu, Hawaii. These nudibranchs are found under rocks in the daytime.

The algae were separated from the host tissue by maceration in a Waring Blender and then in a glass tissue grinder. The macerated material was passed through several layers of cheese cloth to remove large fragments and centrifuged several times to separate algal and animal tissue. All preparations used 0.45 μ Millipore filtered Instant Ocean made with deionized water. Cultures of these isolated zooxanthellae were initiated by inoculating 10-ml tubes containing filter sterilized (0.22 μ) ES enrichment medium (Provasoli, 1966) made with Instant Ocean. Cultures were maintained in an incubator at 25° C with a 12 hr light (28 μ Einsteins m⁻² sec⁻¹: cool white fluorescent lamps), 12 hr dark cycle. Periodic inspections insured that all cultures were unialgal. After several transfers, over a period of at least four months, one-liter flasks containing 600 ml of medium were inoculated with zooxanthellae to obtain sufficient quantities of algae. These cultures were aerated with filtered air and maintained in a light cabinet at ambient temperature, 22–25° C) on a 12 hr light (47 μ Einsteins m⁻² sec⁻¹: cool white fluorescent lamps)/12 hr dark cycle.

Aposymbiotic Aiptasia

Specimens of *Aiptasia pulchella* were obtained from the wet tables at the Pacific Biomedical Research Center, Honolulu. Aposymbiotic polyps were obtained by maintaining them in a darkened 30-liter aquarium in sea water. These bleached animals were fed twice weekly with *Artemia* nauplii hatched in 0.45 μ Millipore filtered Instant Ocean. Two series of experiments were performed with different host material.

Animals used in the first series were kept in the dark for two and a half years. From the time the animals were removed from the dark until the termination of this study, a period of 10 months, none of the *Aiptasia* polyps that were not exposed to algae in the course of the experiments showed any sign of algal infection. Light microscopical study of serial thin sections of these bleached animals showed no trace of any inclusions resembling zooxanthellae.

Because of the small number of these animals available for the experiments, a second series of animals was used. These polyps were from the same source as the animals used in the first series. They were rendered aposymbiotic by maintaining them in the dark for four months. The other conditions were identical. Due to the shorter time without light the bleaching was less successful in this series.

Polyps were maintained in individual 30-ml (first series) or 50-ml (second series) beakers with clear plastic covers. Within each series, polyps were assigned to each of the treatments using a random number table. This was done to remove bias from the initial tentacle numbers and weights. There were no significant differences in tentacle numbers between experimental groups within each of the two series. ($P > 0.5$ and $P > 0.1$ respectively).

Infection

Algae were collected from the flasks and concentrated from the medium by centrifugation. The pellet (about 1–3 ml) was washed several times with 0.22 μ Millipore filtered Instant Ocean. The algae were then mixed with a suspension of ground and filtered *Artemia* nauplii and the mixture was gently centrifuged. After centrifugation, plugs of the pellet were sucked up with a Pasteur pipette and placed on the oral disk of experimental polyps randomly selected from those to be used. In all cases this plug was at least partially swallowed. The *Aiptasia* generally regurgitated some of the ingested mixture within 6 to 12 hr and this material was removed. In each series, controls consisted of uninfected polyps treated in all other ways like the experimental animals. In addition a series of polyps ("normal controls") which had never been bleached were similarly maintained to serve as an estimate of normal growth under the experimental conditions. The initial mean number of tentacles for these normal unbleached controls (12.25 ± 0.71) was between the starting means for the first and second series (9.83 ± 7.93 and 22.37 ± 4.69 respectively).

Maintenance and measurement

The polyps were kept in their individual covered beakers during the course of the experiment. The animals were fed live *Artemia* nauplii hatched in 0.45 μ filtered Instant Ocean once a week. Each polyp received about 40–60 *Artemia* nauplii per feeding. While exact numbers of nauplii were not obtained, efforts were made to normalize the ration. Densities of nauplii were visually adjusted so that each portion was approximately the same. In addition the sequence of feeding was altered each week so that no systematic bias in feeding would occur. It was felt that more precise methods would be fruitless since larger animals had greater catching ability due to longer and more numerous tentacles. Furthermore, the relative amount of food would be smaller for the larger polyps. Also, individual nauplii varied in age and hence yolk content. Finally, in many cases an *Artemia* nauplius would be killed by contact with nematocysts of an *Aiptasia* polyp and fall to the bottom of the beaker where it would remain uneaten. The day after feeding each beaker was cleaned by swabbing the bottom and sides with a disposable cotton applicator to remove detritus and regurgitated or uneaten food, and the 0.45 μ filtered Instant Ocean was changed.

Light was provided by keeping the animals on a shelf next to a window to insure a wide spectral composition. Light values ranged from 15 μ Einsteins m^{-2}

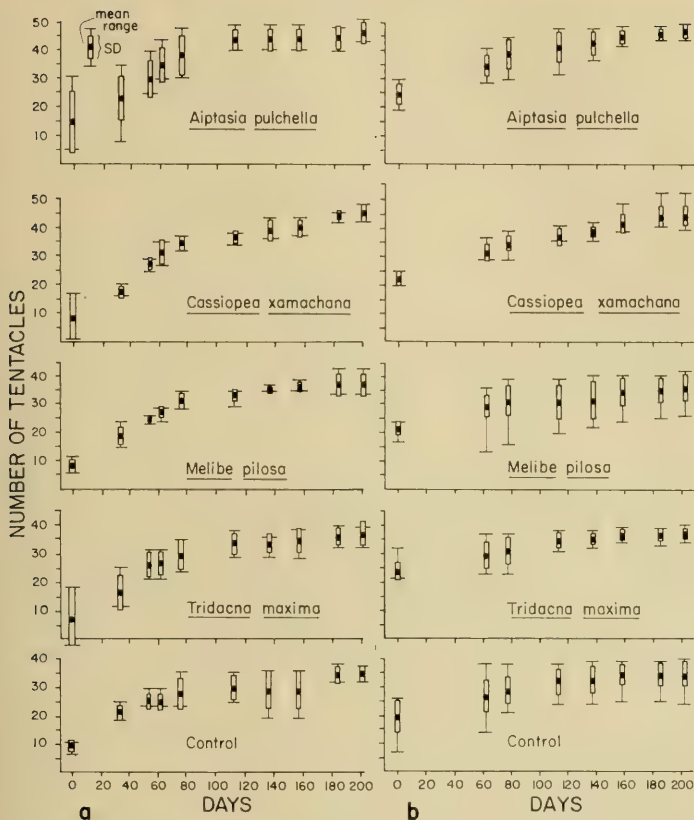


FIGURE 1. The increase in the number of tentacles with time of the first, (A), and second, (B) series of experiments. Means, ranges and one standard deviation on either side of the mean are given. Controls are bleached polyps that were not experimentally infected with zooxanthellae.

sec^{-1} in the morning to $150 \mu\text{Einstein m}^{-2} \text{sec}^{-1}$ in the afternoon. Temperature varied from 22 to 25°C . At approximately 14-day intervals, growth was measured by counting the number of tentacles of each individual. Experiments were allowed to proceed for over 200 days and observations on state of infection, and general health of the polyps were recorded.

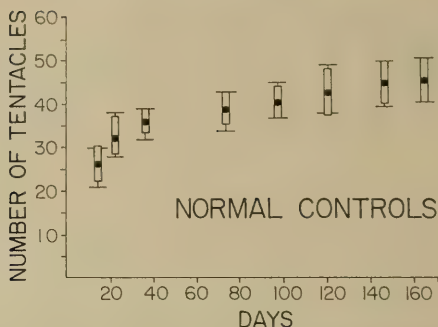


FIGURE 2. The increase in the tentacle numbers of normal control polyps which had never been bleached. These polyps were obtained from the same source as the experimental and control polyps. Since there was no initial infection, the numbering of the days is arbitrarily set at the time when the polyps were isolated.

RESULTS

All of the experimental animals were successfully infected. The polyps became visibly brown within three weeks. In general, the color was first noted in the tentacles. Gradually, the entire body assumed the normal brown coloration.

The results of the tentacle measurements are given in Figures 1 and 2. All the animals in both series, including the normal unbleached controls, exhibited an initial period of increase in tentacle number followed by a more or less asymptotic phase. There are three ways that the growth of the polyps infected with different strains of zooxanthellae could differ: in the final maximum tentacle number; in the rate of increase of tentacles; or in both. To evaluate the first possibility, the

TABLE I

Number of tentacles of *Aiptasia* polyps at termination of experiment (202 days) (mean $\pm$ one standard deviation).

Source of algae	Series 1				Series 2			
	$\bar{X}$	s.d.	n	range	$\bar{X}$	s.d.	n	range
<i>Aiptasia</i>	46.25 $\pm$ 3.95		4	43-51	47.17 $\pm$ 2.79		6	44-50
<i>Cassiopea</i>	45.00 $\pm$ 3.00		3	42-48	44.75 $\pm$ 3.81		8	40-53
<i>Melibe</i>	37.50 $\pm$ 4.04		4	34-43	36.00 $\pm$ 4.93		8	26-42
<i>Tridacna</i>	38.75 $\pm$ 4.03		4	34-41	36.60 $\pm$ 2.07		5	34-39
Uninfected controls	36.00 $\pm$ 2.94		4	33-39	35.70 $\pm$ 3.06		10	30-40
					(34.00 $\pm$ 3.24)		5	30-39)*

Normal controls 45.75 $\pm$ 4.99 4 41-51

* Of the ten controls in Series 2, five showed some sign of infection by the last day though only two were visibly infected till that time. The means of the five remaining aposymbiotic are given here; the five showing any traces of infection were not different. ($P > 0.05$)

Analysis of Variance of Final Tentacle Number (Day 202).

Source of variation	Series 1				Series 2			
	df	SS	MS	F	df	SS	MS	F
Among treatments	4	322.2	80.68	5.98**	4	805.2	201.30	15.56**
All infected vs. control	1	101.4	101.4	4.21*	1	250.07	250.07	8.55**
Mollusks vs. Coelenterates	1	215.4	215.4	16.61**	1	541.5	541.5	35.87**
<i>Aiptasia</i> vs. <i>Cassiopea</i>	1	2.68	2.68	0.21 NS	1	12.6	12.6	0.91 NS
<i>Melibe</i> vs. <i>Tridacna</i>	1	3.13	3.13	0.19 NS	1	0.29	0.29	0.02 NS
within	14	188.5			30	413.0		
Total	18	510.7			34	1,218.2		

results of the different treatments were compared using the number of tentacles on day 202 as the final maximum tentacle number for the polyps. The mean tentacle numbers and statistical comparisons are given in Table I. Because the polyps in each series did not differ in initial tentacle number, the differences in final tentacle number can be attributed to the different treatments. The final tentacle number is markedly greater for polyps infected with zooxanthellae from *Aiptasia* and *Cassiopea*. Neither of these isolates differ significantly in their ability to contribute to the growth of the polyps. Zooxanthellae from *Melibe* and *Tridacna* do not differ from each other, nor do they differ from the uninfected controls, in their ability to support tentacle growth in *Aiptasia*.

The strong agreement between the final tentacle number for polyps infected with each strain in the first and the second series suggests that the possible residual homologous zooxanthellae in the "bleached" polyps in series two did not influence growth. When the bleached controls from the second series that remained visibly uninfected at the end of the experiment were compared with those showing any signs of infection at all, no difference in tentacle number could be found ($t = 2.05$; $P > 0.05$). In addition, the mean number of tentacles for the controls in series one and two was not different ($t = 0.17$; $P > 0.5$), again indicating that the slight degree of infection in the second series did not influence the final tentacle counts. This could be due to the very small number of homologous algae remaining after four months in the dark or to the poor condition of these remaining algae.

To determine if the results were the effects of the infection with different algal isolates or if they were due to the conditions of maintenance, a comparison was made between the experimental animals and the unbleached normal controls which were treated in every other way like the experimental animals. Although the initial tentacle number of these normal controls was between that of the two series, the final tentacle number was not different from the polyps infected with zooxanthellae from *Aiptasia* and *Cassiopea* ($t = 0.01$, $P > 0.95$ first series; $t = 0.02$, $P > 0.95$ second series). This suggests that the experimental infections with these two strains contributed as much to growth as would be expected in a normal polyp kept under the same conditions.

The other aspect of growth that was compared was the rate of increase in number of tentacles. There are two ways that the different treatments could result in the observed differences in final tentacle number. First, the polyps could add tentacles at similar rates but some groups could continue to grow for

TABLE II

Comparison of $T_{\max}$ and r for tentacle counts on experimental polyps.

Source of Algae	1st series		2nd series	
	$T_{\max}$	r	$T_{\max}$	r
<i>Aiptasia</i>	48.92 $\pm$ 3.36	0.014 $\pm$ 0.0037	50.79 $\pm$ 3.27	0.010 $\pm$ 0.0028
<i>Cassiopea</i>	43.71 $\pm$ 1.50	0.020 $\pm$ 0.0042	70.13 $\pm$ 1.65	0.003 $\pm$ 0.0004
<i>Melibe</i>	39.31 $\pm$ 1.17	0.015 $\pm$ 0.0017	37.86 $\pm$ 3.38	0.010 $\pm$ 0.0052
<i>Tridacna</i>	39.41 $\pm$ 2.21	0.017 $\pm$ 0.0036	37.85 $\pm$ 1.89	0.012 $\pm$ 0.0048
Uninfected control	36.12 $\pm$ 1.46	0.016 $\pm$ 0.0028	40.45 $\pm$ 4.36	0.007 $\pm$ 0.0031

Normal control $T_{\max} = 47.36 \pm 3.14$ $r = 0.015 \pm 0.0073$

a longer period. Second, the rates of increase could differ, but the period of growth could be the same. The graphs in Figure 1a and b do not permit resolution of these two alternatives. In order to obtain growth rates, the tentacle counts were fitted to a rearranged von Bertalanffy growth equation (von Bertalanffy, 1938). The form used was $T_t = T_{\max} - G_e^{rt}$. In this form r is the growth rate, $T_{\max}$ is the estimated asymptotic maximum tentacle number, and G is a constant greater than zero. Tentacle counts were fitted to this model using the BMDP P3R non-linear regression procedure.

The results are given in Table II. Two things are apparent from this table. First, with the exception of the polyps infected with zooxanthellae from *Cassiopea*, the $T_{\max}$ values are very close to the actual mean tentacle numbers used in the earlier analysis. This is important because the estimation of $T_{\max}$ utilizes all the measurement data while the mean final tentacle number discussed earlier uses only the last day's count. The agreement between the number of tentacles on day 202 and the $T_{\max}$ value validates the earlier conclusions. The exceptionally high $T_{\max}$ value for the polyps infected with zooxanthellae from *Cassiopea* will be discussed below. Second, there are no significant differences between the rates of tentacle increase in any of the experiments or the controls. This suggests that the polyps will all add tentacles at a similar rate. Since those infected with algae from *Aiptasia* and *Cassiopea* and the normal controls achieve higher numbers of tentacles, they must add tentacles over a longer period than uninfected polyps or polyps infected with zooxanthellae from *Melibe* or *Tridacna*.

There are several other measures of the ability of the different strains to enhance the metabolic activities of the experimentally infected *Aiptasia* polyps. A summary of these is given in Table III. The final dry weights of the polyps infected with zooxanthellae from *Aiptasia* and *Cassiopea* are greater than the uninfected controls and the polyps infected with zooxanthellae from *Melibe* or *Tridacna*, (first series $t = 7.46$, $P < 0.001$; second series $t = 4.86$, $P < 0.001$). While initial dry weights of the experimental animals could, of course, not be obtained, the random assignment of polyps within each series to the experimental groups should have equalized the starting weights. The fact that this randomization resulted in no within-series differences in initial tentacle number supports this assumption. In addition, it can be shown that tentacle number is correlated with dry weight. When the final tentacle numbers and the dry weights of polyps in all the

TABLE III

Condition of polyps at end of experiment.

Source of Algae	1st Series			2nd Series		
	Dry wt. mg	No. of pedal lacerates	State of infection	Dry wt. mg	No. of pedal lacerates	State of infection
<i>Aiptasia</i>	4.0 $\pm$ 1.0	0.25/ind	All completely infected	3.0 $\pm$ 0.6	0.88/ind	All completely infected
<i>Cassiopea</i>	2.6 $\pm$ 1.0	1.0/ind	All completely infected	1.7 $\pm$ 0.3	1.33/ind	All completely infected
<i>Melibe</i>	0.6 $\pm$ 0.2	0.33/ind	1 starting to bleach: others pale	1.5 $\pm$ 0.7	0/ind	Six polyps partly bleached two infected
<i>Tridacna</i>	1.2 $\pm$ 0.7	0/ind	All completely infected	1.2 $\pm$ 0.5	0/ind	All completely infected
Uninfected controls	0.7 $\pm$ 1.0	0.40/ind	None infected	1.1 $\pm$ 0.5	0/ind	5 completely bleached 1 completely infected 4 partly infected
Normal control	2.3 $\pm$ 1.6 mg	2.5/ind	All completely infected.			

experimental groups are compared, correlation coefficients of 0.91 and 0.77 are obtained for the first and second series respectively. A group of *Aiptasia* polyps obtained from the same original source but not kept in beakers also showed a correlation between tentacle number and dry weight ($r = 0.91$). The rate of production of new polyps by pedal laceration is too irregular to allow any unequivocal statements to be made, though it appears that none of the experimental groups produced new polyps as frequently as the normal controls. Polyps infected with zooxanthellae from *Cassiopea* produced the most offspring of the experimental groups in both series.

The final point made by the information in this table is the instability of the infection with zooxanthellae from *Melibe*. In both series, particularly in the second, the polyps infected with algae from *Melibe*, after an initial complete infection, began to lose their algae. This phenomenon was also observed in previous preliminary experiments and seems to be a reproducible phenomenon (cf. Muscatine *et al.*, 1975; Kinzie *et al.*, 1977).

DISCUSSION

While the possibility of the existence of different strains of zooxanthellae has been suggested on indirect grounds for some time, Schoenberg's (1976) work clearly demonstrated that several strains could be differentiated by isozyme electrophoresis, and perhaps more importantly, they would be differentiated by the ability

of the different strains to grow in experimentally infected *Aiptasia* polyps. The results presented here show for the first time that not only do these strains of zooxanthellae exist, but also that they differ in their ability to enhance the growth of the host. Similar results have been reported for the flatworm alga association (Taylor, 1971b; Provasoli *et al.*, 1968). The importance of these differences in the most widespread and important symbiosis—between zooxanthellae and their invertebrate hosts—has far-reaching implications.

Comparison between the polyps infected with zooxanthellae from *Aiptasia* and those infected with algae from *Cassiopsea* suggest some interesting possibilities. It appears that polyps infected with homologous algae achieve a somewhat larger biomass but produce fewer offspring than polyps infected with zooxanthellae from *Cassiopsea*. Comparisons between final tentacle numbers in these two experimental infections is more complex. While polyps infected with algae from *Aiptasia* produce only slightly higher tentacle numbers than those infected with algae from *Cassiopsea*, in both series the $T_{\max}$ values resulting from fitting the measurement data to the von Bertalanffy growth equation are markedly different. In almost all the experiments the $T_{\max}$ values are slightly larger than the observed final tentacle number. The only exceptions are the experiments with algae from *Cassiopsea*. In the first series the $T_{\max}$ value is slightly below the final tentacle count while in the second series the $T_{\max}$ value is very much larger. In addition, in this series, the rate value, r , is smaller than in any of the other experiments. The explanation for this is probably that the polyps in the second series had not stopped growing when the experiment was terminated and hence their growth is not adequately described by the model. Inspection of Figure 1b supports this suggestion. These differences between the two "successful" algal strains raises questions about the precise way that zooxanthellae influence their hosts and the possibility that different strains may have qualitative as well as quantitative differences.

Perhaps the most fundamental question is whether the relative success of the different strains is related to a physiological or biochemical relationship between a particular host species and its normal zooxanthellar strain, or whether success is independent of the taxonomic position of the host but instead is a function of the ecological conditions in which the association is found. Schoenberg's (1976) evidence suggests that the answer may encompass both possibilities. Certainly his results do not support a one-to-one host/strain specificity. In fact, of the seven host species where he sampled more than a single individual, four host species had the same algae strain among individuals sampled, as determined by electrophoresis, while three of the hosts had different algal strains in different individuals. The mollusk-coelenterate differences shown in this paper, however, are suggestive of at least some phylogenetic specificity. Schoenberg's data do not suggest any clearly discernible geographical patterns in the distribution of strains of zooxanthellae among hosts.

Since neither simple host specificity nor geographic factors resolve the distributional patterns, a third possibility is that of ecological parameters. To date, no studies have been published on the photosynthetic requirements of different strains of zooxanthellae *in vitro*. Dustan's (1975) data however, suggest that

in vivo differences might exist between associations at different depths with differing light quantity and spectral quality. The necessity of the host's securing the algal symbiont ecologically suited for the site of settling has been suggested as a reason for maintaining open life cycles, where the host must become infected anew each generation (Kinzie, 1974).

It would not be unexpected if the final answer was in fact a combination of phylogenetic, geographical and ecological effects, but at least the way is now clear for a thorough investigation of this association which is such a dominant feature of tropical reef systems and a paradigm of symbiosis.

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SUMMARY

1. A method is given enabling the differential effects of different strains of zooxanthellae on host growth to be assessed. This technique uses the increase in the number of tentacles as the measure of growth.

2. Aposymbiotic polyps of the anemone *Aiptasia pulchella* reinfected with strains of *Symbiodinium microadriaticum* isolated from the anemone *Aiptasia pulchella* and the scyphozoan *Cassiopea xamachana* grow as well as normal *Aiptasia* polyps.

3. Aposymbiotic *Aiptasia* polyps reinfected with zooxanthellae from the gastropod *Melibe pilosa* and the clam *Tridacna maxima* grew no better than polyps lacking zooxanthellae.

4. These results lead to the conclusion that strains of zooxanthellae differ in their ability to enhance growth of *Aiptasia* polyps under the experimental conditions and that these differences may have important ecological consequences.

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PATTERNS OF MOLTING FREQUENCIES IN CORAL-DWELLING STOMATOPOD CRUSTACEA

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Arthropods must shed the exoskeleton in order to grow. The intermolt interval is usually considered to be the time required to accumulate the reserves for growth at the next molt. This interval sometimes varies with temperature, feeding conditions, injury, seasons, or reproduction. The molting rate decreases with increased age and body size in a number of Crustacea (Passano, 1960; Kurata, 1962). At the extreme of this continuum, some Crustacea have a terminal molt at sexual maturity (Tessier, 1935; Drach, 1939; Carlisle, 1957; Van Engel, 1958; Hartnoll, 1963). Although a number of workers have examined the environmental, physiological, and ontogenetic determinants of the intermolt interval within species, few if any studies have examined patterns of molting frequencies among related species.

Coral-dwelling mantis shrimp provide a particularly good opportunity to examine ecological and evolutionary patterns in molting. Large numbers of species of stomatopods occupy similar coral habitats around the world. As many as seven closely related, morphologically similar species may co-occur in a single tide-flat, allowing simultaneous comparisons of their molting rates. Stomatopods are among the most aggressive crustaceans, and are capable of hammering and stabbing to death other stomatopods as well as various hard-bodied prey (Caldwell and Dingle, 1975). Stomatopods kill unprotected molting individuals (Reaka, personal observation; Serène, 1954; Pyne, 1972). Rapid hardening after molting, molting synchrony, hiding and sealing the burrow entrance with pebbles during molting appear to reduce this danger (Reaka, 1975a, 1976). In spite of the metabolic cost and the risk of mortality at molting, increased body size may confer greater smashing strength, dominance in agonistic encounters, and increased number and size of eggs. An overview of life history patterns in this guild is provided elsewhere (Reaka, 1979), but this study compares several different measures of molting frequencies not examined there, provides a detailed analysis of factors that may affect these molting frequencies along with a detailed treatment of numbers of molts among individuals of different body sizes, and specifically discusses the ecological and evolutionary implications of molting rates in crustacean lineages.

MATERIALS AND METHODS

Methods and conditions of maintenance for the species of stomatopods discussed in this paper are detailed elsewhere (Reaka, 1975a, 1975b, 1976). Coral-dwelling stomatopods were collected in the Gulf of California, Hawaii, Marshall Islands (Enewetak), the Gulf of Thailand (Sattahip), Andaman Sea (Phuket, Thailand), and Australia (Townsville); species, maintenance time, and number of individuals

are indicated in Table I. Specimens of *Gonodactylus zacae*, *G. falcatus*, *Pseudosquilla ciliata* and the species from Enewetak and Australia were maintained, frequently simultaneously, in individual containers at Berkeley except for brief periods at their collection. Maintenance temperatures at Berkeley were not rigorously controlled, but they approximated temperatures recorded in the field at the various study sites (extremes of 19–26° C, but usually 22–24° C). Field and laboratory maintenance temperatures in Thailand were 26–27° C. The species in Thailand were maintained for a shorter period of time than those from other areas; species from Phuket were maintained simultaneously and provided especially reliable comparisons of molting rates among species. All species were fed fish, crustaceans, and molluscs twice or thrice weekly. All individuals were checked for molts and eggs daily or every two days. The exuvia are not eaten for several days, and it is unlikely that molt records were missed.

RESULTS

Estimates of intermolt intervals

It was possible to obtain several different estimates of intermolt intervals in a number of species (Table I). Relatively few individuals molted twice during maintenance. The interval between two molts (MM) was probably unusually short relative to that of most of the population because maintenance time precluded the second molt of individuals with longer intervals.

The period between collection and the first molt of individuals (CM) provided an independent estimate of the length of interval between molts. This estimate included those individuals that molt more slowly and molt only once during maintenance. The CM values were larger than the MM values of the rapidly molting individuals (Table I). However, the period between collection and the first molt included very short intervals from individuals that molt only a few days after collection, and represented only part of the molt cycle of all molting individuals. CM probably underestimated the normal molting interval for individuals in the population.

The third estimate of intermolt interval (CMX) included the period from collection until the individual either molted, was killed, or died. This estimate incorporated the longer intervals of nonmolting individuals. CMX usually exceeded the CM estimate (Table I), but was limited by the maintenance period of the group. Where CMX was equal to or less than CM, a number of individuals had been sacrificed after being maintained less than or about the length of the CM interval (for example, some species from Australia and Enewetak). CM and CMX became less useful where intermolt intervals were long (for example, small species) and where maintenance time approximated or was less than the length of intermolt intervals (for example, species in Thailand).

A fourth method of estimating the average intermolt interval, the number of stomatopod maintenance days 'molt' (DM), was derived from the sum of the days that individuals were maintained, divided by the total number of molts in the captive population. This estimate averaged the effects of fast, slow, and nonmolting individuals and provided a longer molt interval than any of the other three methods (Table I). DM was comparable among groups maintained for long

TABLE I

Number of days stomatopods were maintained (SMD), time between two molts (MM), time from collection until first molt (CM), time from collection until individuals either molt, are killed, or die (CMX), and number of stomatopod maintenance days per molt in the captive population (DM). The data were approximately normally distributed so that means ($\bar{X}$) and standard errors (s.e.) could be used.

Species	SMD			MM			CM			CMX			DM
	$\bar{X}$	s.e.	N	$\bar{X}$	s.e.	N	$\bar{X}$	s.e.	N	$\bar{X}$	s.e.	N	
Baja California													
<i>Gonodactylus zaca</i>	186	4	205	89	6	22	100	5	123	130	5	205	260
Hawaii													
<i>G. falcatus</i>	212	8	224	94	8	41	136	7	136	148	6	224	250
<i>G. hendersoni</i>	264	—	2							264	—	2	527
<i>Pseudosquilla ciliata</i>	187	12	36	102	9	6	108	8	29	113	10	36	205
Florida													
<i>P. ciliata</i>	148	29	29	58	—	4	99	14	18	116	13	29	195
Australia													
<i>G. falcatus</i>	106	16	17				87	10	13	75	9	17	128
<i>G. graphurus</i>	104	11	54	88	—	3	108	10	19	90	10	54	249
<i>G. smithii</i>	138	36	7				69	—	4	128	34	7	242
<i>G. affinis</i>	122	12	42				109	24	7	118	12	42	716
<i>G. hendersoni</i>	176	10	13				142	—	4	164	11	13	572
Enewetak													
<i>G. smithii</i>	99	13	9				92	—	3	87	10	9	296
<i>Haptosquilla glyptocercus</i>	145	4	26				114	4	12	131	5	26	289
Sattahip													
<i>G. falcatus</i>	21	1	38										262
Phuket													
<i>G. chiragra</i>	26	2	345										294
<i>G. viridis</i>	24	1	417										623
<i>G. ternatensis</i>	35	2	62										217
<i>G. mulatus</i>	25	2	110										691
<i>G. smithii</i>	25	2	87										432
<i>H. glyptocercus</i>	20	2	97										970
<i>P. ciliata</i>	34	2	86										322

and short periods, since it included the frequency of molting in the population rather than intervals between molts, which could be limited by maintenance time.

Since each method of estimating intermolt intervals had advantages and limitations, it was desirable to use all four methods to assess the effects of laboratory maintenance, sex and reproduction, and body size upon molting.

The effect of laboratory maintenance

If laboratory conditions biased molting frequencies, one would expect molting rates to decline in groups of individuals that were maintained for increasing

lengths of time. It was possible to analyze the relationship between total number of molts and total number of stomatopod maintenance days in several species of comparable body size where groups of individuals were collected at different times of the year and maintained for different lengths of time. Spearman rank correlations on data from ten different groups of *G. zoeae*, six of *G. falcatus* (Hawaii), four of *P. ciliata* (Hawaii, Florida), and two of *G. graphurus* showed that the number of molts in the group was highly correlated with the total number of stomatopod maintenance days ($r_s = 0.91$, $P < 0.01$, $N = 22$), indicating that wide variations in molting rates did not occur among groups maintained in the laboratory at different times. Furthermore, molting frequencies did not decline among groups maintained for longer periods (since $Y = 0.05X^{1.07}$, $N = 22$; see Reaka, 1975b, for additional data and discussion). In addition, the percentage of individuals that molted during a given maintenance period was plotted against the mean maintenance time for individuals of each group. This relationship also showed no decline in molts with increasing time of maintenance (Reaka, 1975b). Furthermore, the MM interval did not vary significantly among the groups of *G. zoeae* or among those of *G. falcatus* (Hawaii) that were collected and maintained at different times and different years (Mann Whitney U-tests). Therefore, if temperature, feeding regimes, or seasons of collection exerted any effect upon molting rates in different maintenance groups, it was minor.

Survival in the laboratory did not appear to vary among groups of animals maintained for considerable periods in the laboratory. In *G. zoeae*, individuals that molted lived equivalent lengths of time to those that did not molt (Mann Whitney U-test). In *G. falcatus* (Hawaii), individuals that molted lived longer than those that did not molt ($P < 0.05$, Mann Whitney U-test). This result could indicate either that those individuals living long periods in the laboratory had time to molt, or that individuals that did not molt suffered malfunctions and died. Survival did not vary between males and females of *G. zoeae* or of *G. falcatus* (Hawaii) (Mann Whitney U-tests). Survival was not correlated with body size in *G. zoeae* or *G. falcatus* (Hawaii) (Spearman rank correlation coefficients).

The effect of sex and reproduction

There was no significant difference between males and females in MM, CM, or CMX intervals for *G. falcatus* from Hawaii or Australia, *G. graphurus*, or the other small species from Australia and Enewetak (though numbers of molts in the latter groups were small). In *G. zoeae* MM intervals (but not CM or CMX) were longer for females than males ($P < 0.05$, Mann Whitney U-test; Fig. 1).

In *P. ciliata* (Hawaii) CM intervals (but not other estimates) were longer in females than males ($P < 0.05$, Mann Whitney U-test). Stomatopods in Thailand were maintained too briefly to provide other estimates of intermolt intervals, but no sexual differences in DM were conspicuous, and there were no significant differences in the relative numbers of females and males that molted.

Reproduction could modify the length of the intermolt interval, although females carry the eggs with the maxillipeds rather than attach them to the body. No relationship could be detected between the length of MM or CM intervals to season

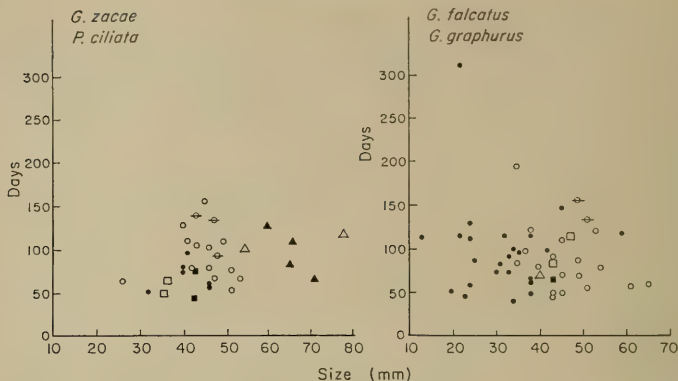


FIGURE 1. Relationship of days between molts (MM) and body size of individuals. In the left graph, specimens of *Gonodactylus zacae* are represented by circles; specimens of *Pseudosquilla ciliata* from Hawaii are represented by triangles and those from Florida by squares. In the right graph, specimens of *G. falcatus* from Hawaii are represented by circles and from Australia by a triangle; specimens of *G. graphurus* are represented by squares. All females are represented by open symbols and all males by closed symbols. Females that brooded eggs between molts are indicated by a horizontal slash through the symbol.

of the year or to month of collection in *G. zacae* and *G. falcatus* (Reaka 1975b, 1976, and above). *Gonodactylus zacae* reproduces from late March to November (personal observations) and *G. falcatus* in Hawaii reproduces year round with peaks in April and October (Kinzie, 1968). There were no significant differences in MM and CM intervals during reproductive *vs.* nonreproductive months in these species (Mann Whitney U-tests). Three female specimens of *G. zacae* laid eggs between molts in the laboratory. The eggs were laid on day 79 of a 134-day interval, on day 57 of a 139-day interval, and on day 47 of a 92-day interval; one female was found with eggs at the eyespot stage (1–2 weeks old) with an exuvium in her cavity in the field. In *G. falcatus* (Hawaii), two females laid eggs on days 86 and 99 of 132- and 154-day molt intervals, respectively. The MM intervals of brooding females were significantly longer than those of nonbrooding females in *G. falcatus* ($P < 0.025$, Mann Whitney U-test), and probably were longer in brooding than nonbrooding females in *G. zacae* ($0.05 < P < 0.10$, Mann Whitney U-test; Fig. 1). Therefore, intermolt intervals generally did not vary between sexes, but where sporadic differences occurred, females showed longer intervals than males. This effect may have been due to lengthened intermolt periods in brooding females.

The effect of body size within species

The relationship between molting intervals and body size within species was tested several ways. There was no significant correlation between MM and body

length of individuals within *G. zoeae*, *G. falcatus* (Hawaii), *P. ciliata* (Hawaii), or *P. ciliata* (Florida) (Spearman rank correlation coefficients; Fig. 1). However, MM intervals were longer in individuals of *P. ciliata* from Hawaii than Florida (Table I, $P < 0.01$, Mann Whitney U-test). Combining both groups, MM intervals were significantly correlated with body length of individuals ($r_s = 0.65$, $N = 10$, $P < 0.05$, Spearman rank correlation coefficient). This result was complicated by the fact that the Florida population was sacrificed early for other reasons, and maintenance time was significantly less for individuals in the Florida than Hawaii populations ($P < 0.01$, Mann Whitney U-test). The MM intervals from the Florida population may have included only the shorter MM intervals, while both shorter and longer MM intervals were recorded in the Hawaii population. There

TABLE II

Body lengths (mm) of stomatopods that molted compared to body lengths of the captive population. Body lengths were not always normally distributed, so medians and ranges are given.

Species	Length of individuals that molted			Length of individuals in captive population		
	Median	Range	N	Median	Range	N
Baja California						
<i>G. zoeae</i>	41	24-59	123	42	15-59	205
Hawaii						
<i>G. falcatus</i>	40	12-65	136	39	8-65	224
<i>P. ciliata</i>	69	45-90	29	68	45-90	36
Florida						
<i>P. ciliata</i>	51	36-62	18	55	36-73	29
Australia						
<i>G. falcatus</i>	45	35-51	13	46	35-51	17
<i>G. graphurus</i>	50	28-70	19	51	27-71	54
<i>G. smithii</i>	66	48-85	4	61	31-87	7
<i>G. affinis</i>	30	26-31	7	28	12-36	42
<i>G. hendersoni</i>	23	20-25	4	22	14-29	13
Enewetak						
<i>G. smithii</i>	18	16-33	6	23	14-62	108
<i>H. glyptocercus</i>	25	18-30	17	25	15-34	36
Sattahip						
<i>G. falcatus</i>	33	20-38	3	31	16-44	38
Phuket						
<i>G. chiragra</i>	57	11-89	30	55	9-99	345
<i>G. viridis</i>	34	13-49	16	33	7-55	417
<i>G. ternatensis</i>	70	51-84	10	68	19-87	62
<i>G. mutatus</i>	39	35-43	4	32	11-44	110
<i>G. smithii</i>	50	30-57	5	48	11-69	87
<i>H. glyptocercus</i>	29	27-31	2	24	10-36	97
<i>P. ciliata</i>	65	60-83	9	66	39-92	86

was no difference in CM, CMX, and DM intervals between the Hawaii and Florida populations in spite of the difference in body sizes (Mann Whitney U-tests).

Molts did not occur most frequently among smaller individuals of each population, since the body lengths of individuals that molted were not significantly different from those expected from the size distributions in any of the captive populations (Table II).

Furthermore, species were maintained for similar, relatively short lengths of time in Phuket, so that the percentage of individuals that molted could be compared in groups and species of different body size. Two species, *G. chiragra* and *G. viridis*, provided sufficient data for dividing the populations into different groups by body size. In *G. chiragra*, 1 of 17 (5.9%) juveniles molted. Among 345 individuals that had fully developed gonopores and gonopods (here defined as adults), there was no significant difference in number of molts among smaller (20–50 mm) and larger (51–99 mm) individuals; 11/137 (8.0%) of the smaller individuals and 18/191 (9.4%) of the larger individuals molted during the maintenance period. Also, in *G. viridis*, a smaller related species, 1/16 (6.3%) of the juveniles molted. Of 418 adults, there was no difference in molting frequency among small (15–34 mm) and larger (35–55 mm) individuals; 8/224 (3.6%) and 7/177 (4.0%) of the smaller and larger individuals, respectively, of this species molted.

The effect of body size among species

Molting rates varied among species in a pattern that appeared to correspond to typical body size of the species. The number of molts that occurred within a population was directly correlated with the total number of days that the animals were maintained (see above). Since the relationship was linear, the total number of molts/total number of stomatopod maintenance days ($\times 100$) provided an index of the molting rate (MR) that varied, for all practical purposes, between 0.0 and 1.0 and was comparable among different populations and species. However, the MR was a population characteristic, not a composite of individual molting rates, so it was desirable to obtain a measure of the reliability of this index.

In *G. zoeae*, males and females of groups maintained at different times in the laboratory provided 10 different estimates of the reliability of the overall MR for the species. The numbers of molts and stomatopod maintenance days in each of these groups were comparable to those of other species that were less abundant or molted infrequently. The standard error and standard deviation for the groups in this species were 0.039 and 0.116, respectively. The standard error and deviation of the MR in six groups of *G. falcatus* (Hawaii) were 0.030 and 0.074, respectively. The groups of *P. ciliata* from Hawaii and Florida provided four estimates of MR with a standard error and deviation of 0.022 and 0.043, respectively. For *G. chiragra*, the standard error and deviation of the MR for five groups (juveniles, small and large females and males) maintained simultaneously were 0.012 and 0.026, respectively. Therefore, the MR values for populations probably were reliable within approximately 0.01–0.04 MR units in most species.

As discussed above, molting did not decline in populations maintained for considerable periods, and molting rates were at least as high in populations main-

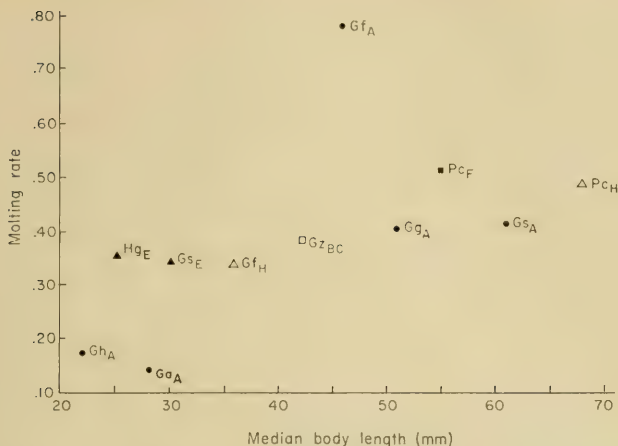


FIGURE 2. The relationship of molting rate (MR) to body size among species. *Gonodactylus falcatus* and *P. ciliata* from Hawaii are represented by open triangles and an H subscript (Gf_H, Pc_H); *G. smithii* and *H. glyptocercus* from Enewetak by closed triangles and an E subscript (Gs_E, Hg_E); species from Australia (*G. hendersoni*, *G. incipiens*, *G. falcatus*, *G. graphurus*, *G. smithii*) by closed circles and an A subscript (Gh_A, Gs_A, Gf_A, Gg_A, Gz_A); and *G. zoeae* from Baja California (Gz_{BC}) and *P. ciliata* from Florida (Pc_F) by open and closed squares, respectively.

tained for long periods as those in populations maintained for short periods (see also Figs. 2, 3). In addition, since there was no effect of body size upon molting frequencies within species, distributions of body sizes within different populations should not have biased results.

Figure 2 portrays the relationship between MR and median body length in populations that were maintained in the laboratory for considerable periods. Molting rate was significantly correlated with the modal, median, mean ($r_s = 0.76-0.84$, $P < 0.01$) and maximum ($r_s = 0.62$, $P < 0.05$) body lengths of individuals in the populations ($N = 12$, Spearman rank correlation coefficients). Figure 3 shows the relationship between MR and median body length in the seven sympatric species from the Andaman Sea (Phuket) and a geographically isolated population from the Gulf of Thailand (Sattahip). The correlation between MR and modal body length (but not other measures of body length) was significant in the eight populations from Thailand ($r_s = 0.71$, $P < 0.05$). Among the seven populations from one tideflat in Phuket that were maintained simultaneously for similar lengths of time, MR was strongly related to the mode, median, mean and maximal body size in the population ($r_s = 0.86-0.96$, $P < 0.01$ in all cases). All data combined (Figs. 2, 3) indicated that larger species exhibited higher molting rates than smaller species ($r_s = 0.62-0.63$, $P < 0.01$ for modal, median, and

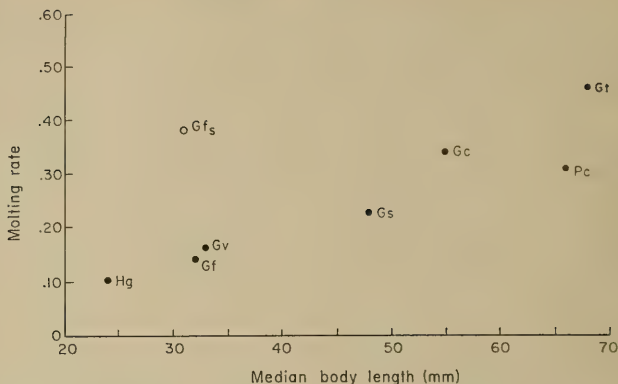


FIGURE 3. The relationship of molting rate (MR) to body size in seven species (*H. glyptocercus*, *G. mutatus*, *G. viridis*, *G. smithii*, *G. chiragra*, *P. ciliata*, *G. ternatensis*) that were maintained simultaneously in Phuket, Thailand (closed circles). Also, one species (*G. falcatus*, open circle) was collected from Sattahip and maintained at Ang Hin on the Gulf of Thailand for a similar period, but at a different time than the other species.

mean body sizes; $r_s = 0.47$, $P < 0.05$ for maximal body size; $N = 20$, Spearman rank correlations).

DISCUSSION

Literature on growth increments is more abundant than that on intermolt intervals in Crustacea because of the difficulty of obtaining information on the latter. Stomatopods provided the opportunity to study molting patterns because they can be maintained easily, with apparently normal behavior and molting, in the laboratory. Furthermore, they provided comparisons of molting patterns in a number of closely related species of different sizes that occupy a single habitat type. Such results, however, must be accepted cautiously because of possible effects of laboratory maintenance upon molting frequencies.

Although it is generally cautioned that animals maintained in the laboratory do not grow as fast as those in natural conditions, experimental and observational results are far from uniform. Various workers have reported that the length of intermolt intervals decreases with increased experimental or seasonal temperatures in well-fed Crustacea (for example, Kurata, 1962; Barnes and Stone, 1974). However, molting in some copepods appears to be temperature independent (Munro, 1974; but see Landry 1975). Also, Broekhuysen (1941) reported no effect of temperature on molting intervals in constantly fed South African crabs in the laboratory; he suggested that more frequent summer molts in the field resulted from increased food availability, rather than from temperature. Some workers found that feeding conditions can alter molting frequencies of some

Crustacea (for example, Kurata, 1962; Crisp and Patel, 1960; Dall, 1965; Choe, 1971), but others found no effect of feeding or diet on molting frequencies except under conditions of extreme starvation and stress; instead, growth increments sometimes may be affected by food deprivation (Thomas, 1965; Lasker, 1966; Stewart and Squires, 1968). Alikunhi (1967) suggested that growth increments of captive stomatopods may have been larger than those in the field where food was less available. Exceptionally long or short photoperiods may influence molting frequencies, but different workers obtained conflicting results on the direction of the effects (Kurata, 1962; Bliss and Boyer, 1964; Stewart and Squires, 1968; Aiken, 1969; Pradeille-Rouquette, 1968). Restrictive and crowded conditions decreased molting frequencies in lobsters (*Homarus americanus*) not acclimated to captivity (Stewart and Squires, 1968). However, Norway lobsters (*Nephrops norvegicus*) reared in the laboratory show similar growth increments to those in the field (Farmer, 1973). Hewett (1974) concluded that growth increments and overall growth rates of captive lobsters (*H. vulgaris*) were normal for the temperatures in which they were maintained. Also, data on American lobsters (*H. americanus*) indicated that adult hatchery-reared lobsters kept in separate tanks at normal seasonal temperatures grow at a rate equal to or greater than wild lobsters, and that molting frequencies are essentially the same as those in the wild (see Templeman, 1948; Wilder, 1953; Hughes and Matthiessen, 1962). These studies show that laboratory results are not always biased, and that, if carefully scrutinized for effects of the above factors, results from laboratory studies can be used to compare patterns of molting in Crustacea.

In the present study, survival and molting patterns showed no conspicuous effects of laboratory maintenance. Molting rates did not decline with increased maintenance time and did not vary markedly among similarly sized species maintained for long (Fig. 2) or short (Fig. 3) periods. The interval between two molts did not differ among different groups of *G. zoeae* or among groups of *G. falcatus* (Hawaii) collected and maintained at different times. Individuals that molted lived as long or longer than those that did not. Survival did not vary between sexes and was not correlated with size in *G. zoeae* and *G. falcatus* (Hawaii). All species and individuals in this study were maintained individually, fed ample food regularly, and were subjected to neither unusually long nor short photoperiods. Variations in photoperiod and temperature were minor, and laboratory temperatures approximated those in the field. Although field and laboratory temperatures for the populations in Thailand were higher than those for the populations maintained in Berkeley, molting rates for stomatopods in Thailand were not higher than for those in Berkeley. Where stomatopods were maintained for only about a month (Thailand), 2-10% of the individuals in different species molted. These figures corresponded to or exceeded the percentage of stomatopods found molting in their holes at various field sites during the equivalent of a month's collecting (personal observations). These points do not rule out the possibility that laboratory conditions influenced molting intervals. However, they suggest that, if results were biased, the effect occurred at collection, could not be detected to progress during the period of maintenance, and should not have selectively influenced a particular group of individuals or species. If there was a laboratory effect upon

intermolt intervals, one would expect the largest individuals and species to be most affected (see Kurata, 1962). However, survival was not correlated with body size within or among species, large individuals continued to molt, and the largest species showed the highest molting rates.

Studies have shown that intermolt intervals lengthen, or molting even may cease, with increasing size or reproductive maturity in many Crustacea. However, Cisne (1973) reported that Ordovician trilobites show a linear rather than a sigmoid growth curve. Additionally, in marine copepods, Miller, Johnson, and Heinle (1977) have documented "isochronal development", where intermolt intervals and percentage growth increments remain nearly constant so that exponential rates of tissue growth continue throughout the life cycle.

In general, there was no correlation between length of the intermolt interval and body size of adult stomatopods in the present study. Possible instances of longer intervals in groups with larger body sizes were not consistent with other estimates of intermolt intervals or could have been affected by maintenance time or reproduction. In species maintained for short, comparable periods in Thailand, the percentage of individuals that molted did not decline among groups of increasing body size in *G. chiragra* or *G. viridis*. Large individuals consistently molted in all of the samples, and body lengths of individuals that molted were not significantly different from those expected from the size distribution of any of the captive populations. Numbers of juveniles were too small to evaluate their molting frequencies here, but intermolt intervals lengthen over larval and early juvenile periods in some stomatopods (Alikunhi, 1967). Although percentage growth increments decreased with increased body size in *G. chiragra* (Reaka, 1975b) and in some American species (R. Caldwell, personal communication), percentage growth increments at the molt did not decline detectably with increased body size in most species of these Indo-Pacific gonodactylids. Therefore, like some copepods, adults of many coral-dwelling stomatopods appear to follow isochronal growth patterns.

Miller, *et al.* (1977) suggested that natural selection can favor isochronal development (rather than lengthening intermolt intervals) when older stages suffer heavy mortality and loss of reproductive value compared to younger stages. Additionally, molting patterns over ontogeny may be related to reproductive demands. As in other Crustacea (Broekhuysen, 1941; Hiatt, 1948; Wilder, 1953), reproduction is associated with lengthened intermolt intervals in adult stomatopods. However, successful reproduction does not occur until relatively late (approximately 50–75% of maximal body size) in stomatopods; delayed reproduction may allow molting and growth to continue over much of the adult life. Regardless of growth and reproduction, continuous molting removes ectoparasites and prevents fouling of the exoskeleton, setae, and gill filaments. Algae, diatoms, protozoa, copepods, barnacles, bivalves, and gastropods have been observed living on the exoskeleton of stomatopods (Boss, 1965a, 1965b; Humes, 1965; Rosewater, 1969; Camp, 1973; Reaka, 1975a, 1978). These organisms are particularly noticeable on individuals in later stages of the intermolt cycle, and stomatopods spend large amounts of time cleaning the exoskeleton, suggesting that epibionts may play an important role in the biology of these species. Also, stomatopods found in the

field frequently bear skeletal wounds, and continuous molting over ontogeny facilitates repair of fighting damage, particularly in large, more aggressive species. These hypotheses illustrate the complexity of selective forces that can act on molting and life history patterns in Crustacea. The diversity of life history patterns in Crustacea, and the factors that mold them, need further comparative study.

Furthermore, within this group of related species, molting frequencies appear to level off at lower rates in small than in large species. These results contrast those expected from *r*- and *K*-selection theory (Pianka, 1970), and portray a continuum between two different responses to a hole-dwelling lifestyle. At one end of the continuum are large species that grow rapidly and accrue advantages in strength. Although the size of the raptorial appendage increases isometrically with body length (Reaka and Gotoff, in preparation), muscle strength increases with cross sectional area (Warner and Jones, 1976), and speed of contraction increases with length of the muscle (Stanley, 1970). Thus, larger individuals and species should be more effective predators and fighters than small ones. Individuals of large species not only molt more frequently, but also show larger percentage growth increments than small species; rapid growth culminates in frequent reproduction with large numbers of large eggs in these species (Reaka, 1979). However, this pattern requires frequent molting with associated mortality; also, large holes—an essential refuge from predators—are less abundant than small ones. At the other end of the continuum, small species with low adult growth rates have few and small eggs (Reaka, 1979), and never achieve the advantages of large size mentioned above. However, mortality associated with molting should be low; crevicular retreats presumably are abundant; and fighting for holes probably is less frequent in small than large species.

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SUMMARY

1. Four estimates of intermolt intervals (time between two molts; time from collection until first molt; time from collection until individuals either molted, were killed, or died; and total number of days stomatopods were maintained relative to total number of molts in the captive population) provided information on molting frequencies in 20 populations of coral-dwelling stomatopods.

2. Number of molts did not decline with increased maintenance time in the laboratory. The time between two molts did not vary among different groups of *Gonodactylus zodiac* or of *G. falcatus* that were collected and maintained at different times. Individuals of *G. zodiac* and *G. falcatus* that molted survived equivalent or longer periods than those that did not molt. Survival did not vary between males and females or with body size in *G. zodiac* or *G. falcatus*. The percentages of individuals that molted during a month of laboratory maintenance in Thailand corresponded to those found in the field.

3. There were no major differences in estimates of intermolt intervals with seasons or between males and females for most species. However, in *G. sacae*, the time between two molts (but not other estimates of intermolt intervals) was greater in females than in males, and *Pseudosquilla ciliata* females showed a longer interval from collection until the first molt (but not other estimates of intermolt intervals) than males. Several females laid eggs during various portions of the intermolt cycle in *G. sacae* and *G. falcatus*; these intervals were lengthened relative to those of nonbrooding females.

4. Molting frequencies did not decline detectably with increased body size of post-juvenile stomatopods. There was no correlation of the interval between two molts and body length of individuals within populations of *G. sacae*, *G. falcatus*, or *P. ciliata*. In Phuket, where individuals were maintained for a relatively short time, the percentage of individuals that molted did not decline among small and large adults of *G. chiragra* or of *G. viridis*. The body lengths of individuals that molted were not significantly different from the distribution of body lengths in any of the captive populations. Thus, stomatopods appear to follow patterns of isochronal growth similar to those found in some copepods.

5. Molting rates were highly correlated with several measures of body size (mean, median, modal, and maximal body lengths) among populations. Large species maintain a high molting rate throughout adulthood, while the molting frequencies of small species level off at lower rates. Ecological and evolutionary factors affecting patterns of molting are discussed.

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THE FEEDING BIOLOGY OF *PODARKE PUGETTENSIS* (POLYCHAETA: HESIONIDAE)¹

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There is little information on feeding biology of hesionid polychaetes, except for the interstitial Microphthalminae (Westheide, 1967). Fauchald and Jumars (1979) believe the Hesionidae are principally carnivorous, and are secondarily coprophagous when commensal, or herbivorous on diatoms when interstitial. Three non-interstitial species of hesionids have been reported to prey on small invertebrates (summarized in Fauchald and Jumars, 1979), but little documentation is available. The three species, two of which have jaws, constitute a small and possibly unrepresentative sample of this large family, which contains about 130 species of which at least 84 have no jaws (Fauchald, 1977). Clearly, the feeding of more hesionids must be examined before confidence can be placed in generalizations about the family.

This study concerns the feeding biology of the hesionid *Podarke pugettensis* Johnson 1901. This species is frequently referred to as *Ophiodromus pugettensis*, as the genus *Podarke* Ehlers was synonymized with *Ophiodromus* Sars by McIntosh (1908); however, its proper taxonomic position is unclear (Fauchald, 1977). This polychaete occurs along the Pacific coast from Canada to Peru (Berkeley and Berkeley, 1948) on hard substrates among the larger epifauna and on rich organic mud (Hickok and Davenport, 1957). Woodin (1968) found a correlation between the occurrence of attached algae (*Ulva* and *Enteromorpha*) and *P. pugettensis* in the intertidal zone at Snug Harbor, San Juan Island, Washington, and this study found large numbers (hundreds m⁻²) of individuals of *Podarke* among attached *Enteromorpha intestinalis* at Garrison Bay, San Juan Island. *Podarke pugettensis* is also a facultative commensal on a variety of asteroids (Hickok and Davenport, 1957; Lande and Reish, 1968), on the apodous holothurian *Protankyra bidentata* (Okuda, 1936), on hermit crabs together with a nereid polychaete (Berkeley and Berkeley, 1948), and with the terebellid polychaete *Eupolymnia heterobranchia* (Woodin, 1968).

Several aspects of the feeding biology of *Podarke pugettensis* were examined: potential and actual diet; rate and degree of digestion and nature of the fecal material; structure and function of the pharynx; and detection and evaluation of potential prey. Woodin (1968) observed that guts of 19 of 20 *P. pugettensis* contained "algae with cellular structure" and that the remaining one contained "a partially digested polychaete and sediment particles." On the basis of this statement, the association of *P. pugettensis* with algae, and the presence of an unarmed pharynx,

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it was hypothesized that *Podarke pugettensis* is herbivorous, ingesting whole algal cells by a pumping mechanism.

MATERIALS AND METHODS

Specimens of *Podarke pugettensis* were collected from the *Enteromorpha intestinalis* mat and underlying mud in the low intertidal zone at Garrison Bay, San Juan Island in Puget Sound, Washington. (However, no specimens of *Podarke* were found at Snug Harbor, the site of Woodin's (1968) study.) Fifty animals were fixed in formalin-alcohol-acetic acid immediately upon collection, and length, width between bases of dorsal cirri at the widest part of the body, and, for 14 animals, volume by displacement were then measured. The gut contents were removed and microscopically examined, and identified to major taxonomic group when possible.

A second set of 50 animals was rinsed to remove adherent material, and was placed in a large container of clean sea water for two days. Fecal material was removed from the container and examined to determine condition (as loose material or pellets) and to identify prey items to major taxa when possible. A third set of 50 animals was held in individual containers for 36 hr, after which the condition of fecal material as pellets or loose material was determined.

For feeding trials, individuals of *P. pugettensis* were held in 50-ml beakers, with temperature maintained at about 10° C by running sea water. Experimental animals were starved several days prior to the tests. Eight worms were held for two days with each of the test food materials: *Ulva* pieces, specimens of *Enteromorpha intestinalis*, and mud from Garrison Bay which had been passed through a 0.5-mm sieve. The algae were rinsed well and examined microscopically to ensure that no small animals adhered to them. At the end of the trials, the animals were fixed and the gut contents examined.

A variety of small invertebrates was offered to approximately 20 specimens of *P. pugettensis*; the incidence and time of feeding were noted; and the feeding behavior was observed. After a polychaete fed, its container was checked irregularly for fecal material, the time of appearance noted, and the material examined microscopically to evaluate degree of digestion.

The muscular structure of the pharynx was examined by dissection and by transverse and longitudinal serial sections of the anterior halves of specimens of *P. pugettensis*. Animals were relaxed in MS-222 (Tricaine methanesulphonate), fired in alcoholic Bouin's fixative for 1 hr, then cut in half transversely to allow better penetration of the fixative, and fixed for 24 hr. Specimens were embedded in Paraplast using conventional techniques, sectioned at 15 μ m, and the sections were stained with Cason's rapid one-step Mallory-Heidenhain triple stain (Humason, 1967). Representative sections were photographed through a microscope.

RESULTS

Potential and actual diet

Items present in the guts or fecal pellets of *Podarke pugettensis* collected at Garrison Bay included remains of polychaetes (in the families Lumbrieridae,

Dorvilleidae, Capitellidae, Orbiniidae, Hesionidae, Nereidae, Spionidae, Terebellidae, Syllidae, and others unrecognizable from the setae); crustaceans (ostracods, harpacticoid copepods, tanaids, and isopods); kinorhynchans; nematodes; chains and individuals of centric and pennate diatoms; one filament of *Enteromorpha*; and some sediment and debris. The gut contents of 50 *Podarke* specimens from the field included 862 recognizable prey items: 851 copepods, generally recognizable as harpacticoid; 8 polychaetes; 1 ostracod; 1 tanaid; and 1 isopod. Harpacticoid copepods thus constituted 98.7% of the identifiable prey items. The range of number of items per gut was 0 to 63 ($\bar{x} = 17.2$, s.d. = 3.9).

All individuals of *Podarke pugettensis* held with *Ulva* or *Enteromorpha*, and five of those held with sieved mud, had empty guts. One of the animals held with sieved mud contained two harpacticoid copepods; one contained one harpacticoid copepod and one foraminiferan; and another contained one nematode. These prey animals had apparently been in the mud, having passed through the sieve.

Specimens of *P. pugettensis* in the laboratory ate the following food items: polychaetes in the families Lambrineridae, Dorvilleidae, Phyllodocidae, Capitellidae, Hesionidae (juvenile *Podarke pugettensis* as prey), and Ampharetidae; tanaids; and Tetra-Min® fish food flakes. Larval echinoids may also be eaten (R. Highsmith, personal communication). Accepted food was always small in size relative to the *Podarke*.

Volume is a better measure of animal size than any simple linear measure because volume is unaffected by changes in shape or state of contraction of a soft-bodied animal. However, volume is very time consuming to determine directly for small animals. The relationship of displacement volume to length and width for individuals of *P. pugettensis*, determined by least-squares linear regression using logarithmic transformation for all variables, in $\ln V = -1.395 + 1.359 \ln W + 1.316 \ln L$, or $V = 0.248 W^{1.359} L^{1.316}$, where V is the volume in mm³, and W and L are the width and length, respectively, in mm. The high correlation coefficient, $r = 0.98$, indicates that length and width can be used to accurately predict volume. This relationship was used to calculate volume of the *P. pugettensis* individuals for which gut contents had been determined but which had not been directly measured for volume.

Number of prey items in the gut versus volume of the specimens of *Podarke pugettensis* is plotted in Figure 1. The null hypothesis that the number of prey items in the gut is independent of predator volume cannot be rejected ($r = 0.10$, $P = 0.48$). Thus, proficiency of prey-catching does not increase with size, and an animal of any size may have an empty gut. However, the maximum number of prey items potentially present in the gut is probably related to the volume of the predator. If prey size is constant and gut volume is a monotonically increasing function of body volume, then maximum number of prey items in the gut, M , would be a monotonically increasing function of predator volume. The seven circled points in Figure 1 are included in this function; the least-squares regression through these points (with standard errors for intercept and slope) is $M = 2.07 (\pm 3.67) + 0.880 (\pm 0.090) V$. A correlation is inherent in these points because of the criterion by which they were chosen; the very high correlation coefficient, $r = 0.97$, indicates a good fit to the straight line (the coefficient for a

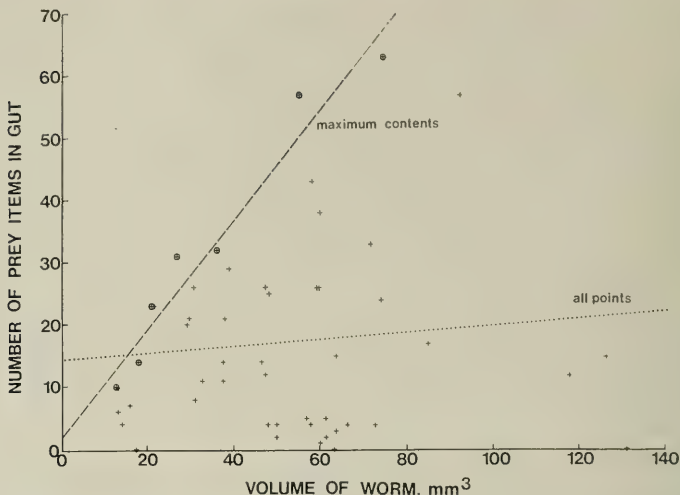


FIGURE 1. Number of prey items in the gut versus volume of field-collected specimens of *Podarke pugettensis*. The dotted line is the linear regression through all the points, which has a slope not significantly different from zero; the dashed line is the linear regression through the circled points, estimating the maximum gut contents.

quadratic term for this regression is not significantly different from zero, $P = 0.14$). This line is a minimal estimate of the maximum number of prey items in the gut of an individual *Podarke pugettensis*; an animal could contain more prey items than this if the prey items were smaller than average.

For each animal the ratio of actual number of prey items to the predicated maximum was calculated as an estimate of gut fullness. The mean fullness was 0.442 (s.d. = 0.353) out of a possible 1; on the average the guts of specimens of *P. pugettensis* were somewhat less than half full. Fullness, F , decreases with worm volume: the least-squares regression (with standard errors for intercept and slope) is $F = 0.713 (\pm 0.097) - 0.0053 (\pm 0.0017) V$; the correlation coefficient, $r = 0.45$, indicates that the slope of this line is significantly different from zero, $P = 0.003$. Calculations of fullness for the five observations with volumes greater than 75 mm³ depend upon extrapolation of the maximum contents line. However, elimination of these five points from the regression does not significantly change the equation: $F = 0.780 (\pm 0.127) - 0.0071 (\pm 0.0026) V$, $P = 0.01$.

Digestion and excretion

Gut passage time is defined as the time between feeding and production of fecal material. This time cannot be determined exactly for each trial, but can be placed

in a definable range, because: animals were sometimes fed more than one prey item over a period of 30 to 60 min, the time at which fecal material was produced is known only as an interval between observations, and animals sometimes produced at separate times two fecal pellets containing recognizable parts of the same prey item. The shortest possible gut passage time for a trial is from the last feeding to the beginning of the interval in which the first fecal material was produced; the longest possible time is from the first feeding to the end of the interval in which the last fecal material was produced. The midpoint of this range was used as an estimate of gut passage time for each trial. Gut passage times for 29 observations ranged from 12.5 to 29 hr ($\bar{x}$ = 18.1, s.d. = 3.75).

The nature of the fecal material *Podarke* produces depends on the type of prey eaten. The 50 animals collected at Garrison Bay and held in a group produced 17 discrete fecal pellets, of which 15 contained polychaete remains; the remaining fecal material was loose. Seventeen of the 50 animals collected at Garrison Bay and held individually produced discrete fecal pellets; the others produced loose material. In the feeding trials, remains of polychaetes were always packaged in a discrete pellet bound together by a homogenous brown substance, while tanaid remains were always deposited as loose material.

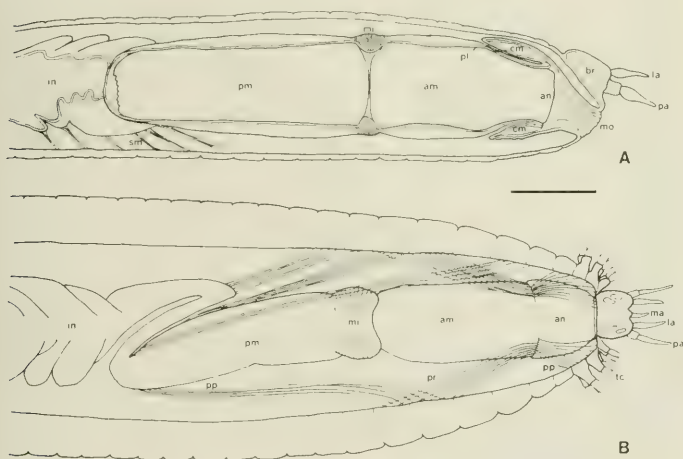


FIGURE 2. Anterior of a specimen of *Podarke pugettensis*, showing structure of pharynx and supporting muscles. A, sagittal section; B, dorsal dissection, parapodia not shown. Scale = 1 mm. Labels: am = anterior muscular region of pharynx; an = anterior nonmuscular region of pharynx; br = brain; cm = circular muscle; la = lateral antenna; ma = median antenna; mi = middle region of pharynx; mo = mouth; pa = palp; pl = pharyngeal lining; pm = posterior muscular region of pharynx; pp = pharyngeal protractor muscle; pr = pharyngeal retractor muscle; sm = suspensor muscle; tc = tentacular cirri.

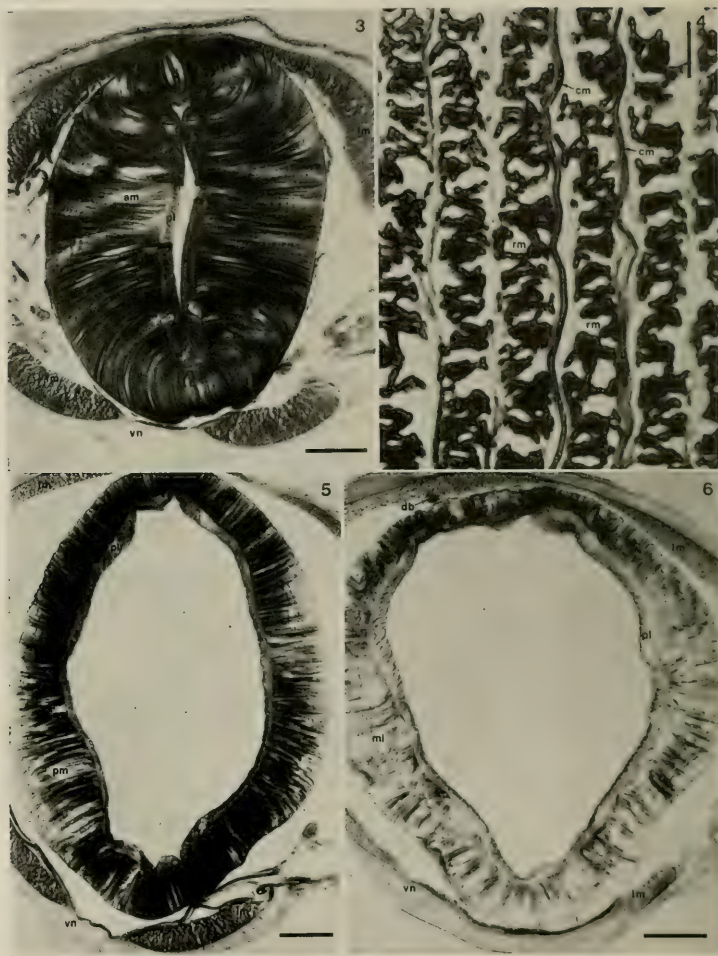


FIGURE 3. Transverse section through the anterior muscular region of the pharynx, in closed position. Note the thickness of the lateral walls and orientation of the radial muscle fibers. Scale = 0.2 mm. Labels as in Figure 2, plus *lm* = longitudinal muscle; *vn* = ventral nerve cord.

In laboratory feeding trials, clean prey polychaetes were fed to specimens of *Podarke pugettensis* in clean beakers, yet the fecal material produced after such a feeding frequently contained not only hard parts of the prey (jaws and setae), but also diatoms and sediment particles. This material must have been in the gut of the prey. Animals appeared to be thoroughly digested before they were egested: only jaws and setae of polychaetes and the exoskeleton of crustaceans remained in the hindgut or fecal material. Plant material appeared undigested: diatoms and *Enteromorpha* in the gut or fecal material were frequently green. Thus, any undigested plant material in the gut of a prey animal remains undigested in the gut or fecal material of a *Podarke*.

Pharynx structure and function

The pharynx of *Podarke pugettensis* is muscular and stiff, has no jaws or teeth, and extends through about 18 segments when not everted. The pharynx consists of four regions: (1) anterior nonmuscular, (2) anterior muscular, (3) middle, and (4) posterior muscular (Fig. 2). Prominent protractor and retractor muscles connect the pharynx to the body wall. No septa are present in the pharyngeal region, and septa are incomplete posteriorly. The pharynx may be everted as far as the middle region for feeding, although extension is not essential for feeding to occur. Eversion appears to be primarily the result of direct action of the protractor muscles, probably supplemented by increased hydrostatic pressure in the coelom from contraction of circular body-wall muscles. Eversion by increased pressure alone would be less efficient because the pharynx is rodlike rather than saclike, and the body is not compartmentalized by septa.

The anterior nonmuscular region consists of a thin, folded membrane which forms a partial sheath over the anterior muscular region (Fig. 2). This sheath extends back over the anterior muscular portion of the pharynx during eversion. The transition between the anterior nonmuscular and muscular regions is formed by a ring of circular muscle, illustrated in Figure 2a. This ring acts as a sphincter valve at the anterior end of the anterior muscular region.

The two muscular regions have extremely thick walls laterally, with the lumen forming a slit between the walls in a relaxed pharynx (Fig. 3). The anterior and posterior muscular regions have identical muscle fiber arrangements. Radial muscle fibers compose the majority of the walls, with circular muscles being much less abundant (Fig. 4). Radial and circular muscles occur in alternate bands; the bands of radial muscle are 10 to 20 μm thick, and those of circular muscle are 2 to 5 μm thick. The radial muscle fibers of the dorsal and ventral walls of the

FIGURE 4. Tangential section through the lateral wall of muscular region of the pharynx showing alternating bands of radial and circular muscle. Scale = 0.02 mm. Labels as before, plus rm = radial muscle.

FIGURE 5. Transverse section through the posterior muscular region of the pharynx, in open position. Notice that the pharyngeal walls are nearly uniform in thickness, compared with Figure 3. Scale = 0.2 mm. Labels as before.

FIGURE 6. Transverse section through the middle region of the pharynx, in open position. Note the uniform thickness and low density muscle fibers of the pharyngeal wall. Scale = 0.2 mm. Labels as before plus db = dorsal blood vessel.

pharynx curve toward the midline as they approach the periphery of the pharynx (Fig. 3). The circular muscles are concentrated near the periphery of the pharynx, and are absent near the pharyngeal lining. When the radial muscles contract, the lateral walls are thinned so that the pharyngeal walls are almost uniform in thickness, and the angle of the folds at the dorsal and ventral edges of the lumen is increased. The lumen is thus opened, achieving a nearly circular cross-section (Fig. 5). Contraction of the circular muscles, with relaxation of the radial muscles, opposes this action, closing the pharynx.

The middle region has walls that are thin relative to the lateral walls of the muscular regions, but relatively uniform in thickness. Circular muscles predominate in this region, and radial muscles are relatively sparse (Fig. 6). The lumen is almost circular in cross-section. The middle region acts as a sphincter valve: contraction of the circular muscles decreases the diameter of the lumen, although it might not be totally blocked. Contraction of the weaker radial muscles opens the middle region to its original diameter.

The pharynx acts as a two-stage pump, with each of the muscular regions providing pumping force, and the valves at the anterior end of each section serving to control the direction of flow by closing and opening in coordination with the opening and closing of the muscular regions. Actual ingestion is a very rapid process, occurring within one second for small prey. The probable sequence of events during ingestion is: the pharynx may be everted partially or completely (as far as the middle region); with the middle and posterior regions closed and the anterior valve open the anterior muscular region opens rapidly, causing an inflow of water and nearby objects into the anterior lumen of the pharynx; the anterior valve closes and the middle region opens, then the posterior muscular region opens as the anterior muscular region closes; this forces the water and food from the anterior to the posterior lumen of the pharynx. Finally, the middle region closes, then the posterior muscular region closes, forcing the food into the intestine.

Large prey items sometimes became lodged in the pharynx, and were then expelled, often with considerable thrashing about and apparent difficulty. After successfully swallowing large prey, specimens of *P. pugettensis* frequently partially everted the pharynx and continued pumping motions, perhaps in an attempt to force the prey from the posterior region of the pharynx into the intestine.

Feeding behavior

In laboratory feeding trials, specimens of *Podarke pugettensis* accepted both living and dead prey of various sizes, whole and in pieces, and also ate flakes of Tetra-Min®. Live, moving prey items were rapidly detected from a distance of at least 3 cm, and directly approached, while nonmoving food items (dead animals and Tetra-Min®) were reacted to only slowly or not at all from a similar distance. When the food was encountered, either as a result of random or directed movements, it was usually investigated by touching with the palps and possibly the antennae, and then eaten or rejected. Occasionally, small moving prey items were ingested without visible contact prior to ingestion: the pharynx was extended close to the prey which was then swallowed without further investigation. Large polychaetes were generally rejected after investigation, while small specimens of the

same species were commonly eaten. When a *Podarke* did attempt to eat a large prey item, ingestion often failed or was incomplete, and the prey was spit out and not approached again. Long polychaetes were sometimes swallowed with multiple pharyngeal pumping motions after partial ingestion or the part of the prey not initially swallowed broke off and was usually ignored afterwards. When *P. pugettensis* encountered dead polychaetes or flakes of Tetra-Min®, the food item was briefly touched by the palps. It was then ingested, usually with minimal eversion of the pharynx, or rejected.

DISCUSSION

The hypothesis that *Podarke pugettensis* is a herbivore has been falsified, at least for the population at Garrison Bay. The diet consists of small invertebrates, primarily harpacticoid copepods. The potential diet is quite similar to the actual diet (see Fauchald and Jumars, 1979, for a discussion of this distinction). Diatoms, algae, and debris found in the gut or feces of *P. pugettensis* (as by Woodin, 1968) are ingested either accidentally, by the suction method of feeding, or indirectly, being in the guts of prey animals.

A knowledge of diet is important for determining the general trophic level and specific position in the food web of any animal. *P. pugettensis* can be classified as a primary or secondary carnivore, consuming a wide variety of small, herbivorous or carnivorous, benthic invertebrates. An estimate of the impact *Podarke* could have on populations of prey animals can be made from estimates of *Podarke* densities and feeding rates and prey productivity rates. Woodin (1968) found densities of *P. pugettensis* in the intertidal zone at Snug Harbor ranging from 30 to 710 m⁻² with an average density of 380 m⁻². Specimens of *Podarke* from Garrison Bay contained an average of 17 copepods in their guts, and had an average gut passage time of 18 hr. An estimate of the field feeding rate is thus 17 copepods per 18 hr, or 0.94 copepods per hr. If this feeding rate were constant throughout the year, one *Podarke* would consume an average of 8300 copepods per year. Copepod weights vary considerably: average dry weights for harpacticoid copepods from North Inlet, South Carolina, are about 2.2 µg and 1.2 µg from mud and sand habitats, respectively (B. C. Coull, personal communication). Dumont, Van de Velde, and Dumont (1975) found the average dry weights of two species of harpacticoid copepods were 1.45 µg and 7.65 µg. The average carbon content of the harpacticoid copepod *Huntelemania jadensis* from Puget Sound, was 3.2 µg for females, 2.3 µg for small males and 6.9 µg for large males (Feller, 1977). Using an arbitrary average of 3 µg carbon per copepod, an individual *Podarke* would consume about 25 mg C yr⁻¹, and a population of 380 *Podarke* m⁻² would consume about 9.5 g C m⁻² yr⁻¹ of copepods. Feller (1977) estimated the average production rate of *H. jadensis* to be about 1 g C m⁻² yr⁻¹. *H. jadensis* is a dominant benthic copepod on Puget Sound beaches, representing an average of 26% of the total number of copepods (Feller, 1977), so total copepod productivity may be on the order of 4 g C m⁻² yr⁻¹ in the intertidal zone of Puget Sound. J. Fleeger (in preparation, Louisiana State University) estimated the average production rate of three species of benthic harpacticoid copepods to be 0.6, 1.0, and 2.9 g C m⁻² yr⁻¹. These three species represented 83% of the total copepod abundance in North Inlet, so total

copepod productivity may be about $5.4 \text{ g C m}^{-2} \text{ yr}^{-1}$. These productivity estimates are approximate and actual values would certainly vary considerably; also, the consumption rate estimated for *Podarke pugettensis* is probably high, as *Podarke* densities and feeding rates may drop in the winter. Nonetheless, it appears that a population of *Podarke pugettensis* could have a significant effect on co-occurring benthic copepod populations.

The fact that the guts of specimens of *P. pugettensis* are on the average less than half full indicates that these worms are not usually able to obtain enough food to fill their guts. The amount of food they consume is apparently limited by the relative prey availability, which is a function of both prey abundance and the ability of *Podarke* to capture prey.

The gut passage time of about 18 hr, which is relatively long for a small carnivore with a straight intestine, may be an adaptation to the infrequent feeding which is reflected in the half-empty guts. This long gut passage time allows the greatest nutrient extraction from a limited food supply. An organism that has an essentially unlimited supply of low quality food available, such as a deposit feeder, may maximize the rate at which it obtains nutrients by having a short gut passage time and only digesting the most available substances (Taghon, Self, and Jumars, 1978). For example, the sediment-surface-feeding spionid polychaete *Boccardia* sp. has a gut passage time of about one hour (personal observation). However, a carnivore such as *Podarke* with a limited supply of high quality prey may maximize its nutrient absorption rate by thoroughly digesting each prey item. Ockelmann and Vahl (1970) found that the mean digestion time of the carnivorous polychaete *Glycera alba* was 22.5 hr at 8°C , and that only about 10% of field-collected animals had food in their guts. The digestion of *Glycera alba* is very efficient, with 78% of the carbon in the prey being absorbed (Vahl, 1976). *Glycera* seems to be even more limited by relative prey availability than *Podarke*, and has a correspondingly longer digestion time.

The pharyngeal pumping method which *Podarke* uses to ingest food works most efficiently when pumping liquid, and thus in ingesting small particles which move readily with the liquid. The anterior end of the pharynx must be very close to the prey so that the prey will be drawn into the pharynx with the flow of water. Thus, the primary function of eversion of the pharynx in *P. pugettensis* is to bring the tip of the pharynx next to the prey. The extreme rapidity of eversion is useful in catching prey that are moving or might move away if *Podarke* approached. However, the thick muscular walls of the pharynx are not very extensible, and so cannot expand to accommodate large prey. This limitation was seen when large prey became lodged in the pharynx. The entire pharynx is adapted for a diet of small, rapidly-moving animals.

On the basis of the behavior of *Podarke pugettensis* relative to various types of food, preliminary conclusions can be drawn regarding the primary sensory modes that *Podarke* uses to detect and evaluate potential prey. The rapid response of *Podarke* to distant, moving prey, compared to the slow or non-response to distant, nonmoving food, indicates that *Podarke* can discern vibration and judge the direction of the prey as a result of the motion. Daly (1973) found that vibration sensing is a primary mode of prey detection in the polynoid *Harmothoe imbricata*, which uses

palps or cirri to sense the vibrations. Oeckelmann and Vahl (1970) found that *Glycera alba* within their burrows utilize vibration receptors in the prostomial and peristomial regions to detect and locate prey moving on the surface of the sediment.

It is possible that *Podarke* uses vision to detect prey motion at a distance, rather than vibration sensing. *Podarke* possesses two pairs of eyes (Fig. 2b) but these are oriented upwards rather than forwards (the rear pair actually points to the rear slightly). Also, vibrations travel through water much more efficiently than light, and vibration detection is not impaired by turbid water or darkness (Horridge, 1966). It appears unlikely that *Podarke* uses its eyes for prey detection.

Prey that are approached and investigated are sometimes rejected, for reasons that are not always clear. One property of food items obviously important to *P. pugettensis* is size: if an item is too large, it cannot be swallowed. The general rejection of large items indicates that *Podarke* can judge the size of prey, apparently by feeling with the palps and antennae. The probability of attempted ingestion appears to be related to the probability of successful ingestion. Ingestion is a stochastic process, the probability of success decreasing as the size of the prey item increases, but also varying with the orientation of the prey. Success is higher if the prey item is approached from the smallest side, as from the end of a cylinder. However, no tendency for *Podarke* to orient relative to elongated prey in any particular direction was observed.

Chemosensory ability is also apparently important to *Podarke pugettensis* in prey detection and evaluation, as individual *P. pugettensis* will ingest dead polychaetes and Tetra-Min® flakes. Detection of vibration is unlikely to be important with these food items, although size selection may occur. Commensal individuals of *Podarke* have been shown (Bartel and Davenport, 1956; Hickok and Davenport, 1957) to locate host asteroids by chemosensory ability.

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SUMMARY

1. *Podarke pugettensis* is carnivorous, ingesting a wide variety of small benthic invertebrates, including members of at least 11 polychaete families. Harpacticoid copepods constituted the bulk of the diet in the field population examined. Food items are limited to those that are small relative to the predator and can fit through

its pharynx. Plant material and sediment are not ingested purposely, but may enter the gut by being sucked in with the prey, or by being within the gut of the prey.

2. Ingested animals appear to be thoroughly digested by the time they reach the hindgut, while plant material appears to be undigested. The gut passage time is generally 12 to 24 hr, indicating a slow movement and thorough digestion of animal material. The long gut passage time may be necessary because *Podarke* is limited in the food it consumes by relative prey availability, and so must digest food as thoroughly as possible.

3. The unarmed pharynx acts as a double-action pump, with two thick-walled muscular regions and two thinner-walled valves. The massive walls of the muscular regions consist mostly of radial muscle fibers which can rapidly open the pharynx, sucking in prey.

4. *Podarke pugettensis* appears to utilize vibration sensing, direct touching, and chemoreception to detect and evaluate potential prey. Size of the prey appears to be the most critical factor influencing prey selection.

5. Calculations of feeding rate of *Podarke* indicate that this predator could consume a significant portion of the production of co-occurring benthic copepod populations.

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PREDATION BY THE ESTUARINE SHRIMPS *CRANGON*
FRANCISCORUM STIMPSON AND *PALAEEMON*
MACRODACTYLUS RATHBUN

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Crangon franciscorum and *Palaemon macrodactylus* are caridean shrimps co-existing in the Sacramento-San Joaquin Estuary of California. *C. franciscorum* is native to estuaries along the northeastern Pacific Ocean, while *P. macrodactylus* is a form introduced from the Orient (Newman, 1963). No previous reports were found which deal with the feeding habits of any species of *Crangon* or *Palaemon* in estuaries along the Pacific Ocean. Investigations of *Crangon* and *Palaemon* species in estuaries and coastal waters elsewhere have reported that some species are carnivorous (Lloyd and Yonge, 1947; Allen, 1960; Price, 1962; Herman, 1963; B. L. Welsh, unpublished Masters Thesis, University of Maryland, 1970; Wilcox and Jeffries, 1974; Subrahmanyam, 1975).

Research on vertical migrations of *C. franciscorum*, *P. macrodactylus*, and a coexisting smaller and much more abundant mysid shrimp *Neomysis mercedis* (Sitts and Knight, unpublished), indicate that the mysid may be an important food resource for *C. franciscorum* and *P. macrodactylus*, just as it is for many smaller fishes of the estuary (Gannsle, 1966). All three shrimps undergo nocturnal vertical migrations (Sitts and Knight, unpublished). Visual estimates of *C. franciscorum*, *P. macrodactylus* and *N. mercedis* biomass indicate that during some seasons *N. mercedis* biomass was equaled or exceeded by the biomass of the other two species. If *N. mercedis* was a major prey of *C. franciscorum* or *P. macrodactylus*, predation might change with vertical migration, and, if the biomasses were similar, the amount of predation could limit mysid abundance. Also, since the mysid is an important trophic resource for fish, predation on *N. mercedis* by the caridean shrimps may affect fish production.

This study was done to quantify and compare the biomasses of *C. franciscorum*, *P. macrodactylus*, and *N. mercedis*, and to determine and compare the feeding habits of *C. franciscorum* and *P. macrodactylus*.

MATERIALS AND METHODS

Shrimp were collected in the main channel of the Sacramento-San Joaquin Estuary of central California (long 121° 54' 50" W, lat 38° 02' 51" N). Details of sampling and the physical and chemical characteristics of the study site are given by Sitts (1978). Simultaneous collections were made of organisms at depths of 1, 5, and 9 m (bottom) about every three hrs over diel cycles on February 25-26, May 6-7, July 19-20, September 17-18 and 23-24, and November 30-December 1 of 1976. The diel samplings will be referred to by month, with the latter three

referred to as mid and late September and late November. Nets with 50-cm-diameter mouths and #8 (200- μ m) mesh were used. Samples were preserved in the field in 5% formalin, then, within seven days, the preservative was changed to 70% ethanol. All specimens of *C. franciscorum* and *P. macrodactylus* with carapace lengths >2 mm were counted and measured. All specimens of *N. mercedis* were counted and measured except when large catches necessitated subsampling.

Length vs. dry-weight equations were obtained for fresh specimens of each species. Lengths of individuals were determined to the nearest mm under a 10 $\times$ dissecting microscope. Total lengths of *N. mercedis* specimens were measured from the anterior of the eyes to the anterior of the telson. Specimens of *C. franciscorum* and *P. macrodactylus* were measured for shortest dorsal carapace length from the anteriodorsal midpoint between the posterior edges of the eye sockets to the posteriodorsal edge of the carapace. Dry weights were measured after live lengths were obtained and organisms were dried at 50° C for ≥ 36 hrs, a period assumed sufficient for complete drying. Seventy-five specimens of *N. mercedis* were weighed to the nearest μ g on a precalibrated electrobalance. Fifty-five specimens of *C. franciscorum* and 30 specimens of *P. macrodactylus* were weighed to the nearest 0.01 mg on a precalibrated precision balance. Additional unpublished length and weight data for *N. mercedis* specimens were available from an earlier study (Simmons and Knight, 1975). Since these data fit well with the present data at similar lengths, they were incorporated into the analysis to extend the length-weight relationship.

Live lengths did not differ significantly (t -test, $\alpha 0.05$) from preserved lengths for 28 *N. mercedis* individuals and 15 *C. franciscorum* forms measured live then preserved for seven days in 5% formalin followed by seven days in 70% ETOH. *P. macrodactylus* was assumed also to be unchanged by preservation.

All specimens of *C. franciscorum* and *P. macrodactylus* in each field sample were measured. Up to 75 specimens of *N. mercedis* were measured in each sample. If over 150 specimens of *N. mercedis* were in a sample, 75 individuals were selected randomly from each aliquot of the entire sample. In samples with 76 to 150 mysids, 75 were measured in perpendicular sweeps crossing near the middle of the tray. The proportion of individuals of *N. mercedis* in each size class relative to the total number of individuals measured was extrapolated to the number of individuals of each size per m^3 . Then, the dry weight (mg) per m^3 was calculated for each size class. The dry weight (mg) for each specimen of *C. franciscorum* and *P. macrodactylus* in a sample was calculated, followed by estimates of the mean dry weight (mg) per individual and per m^3 .

Foregut contents of up to five specimens of *C. franciscorum* and five specimens of *P. macrodactylus* from each sample were examined. In samples with more than five specimens of *C. franciscorum* and five specimens of *P. macrodactylus*, five individuals of each species were chosen randomly. Carapace lengths were measured, and foreguts were removed intact, placed on a depression slide, and teased open. Foregut contents were removed with dissecting needle to water in the depression, and spread. Recognizable contents were identified and enumerated. Foregut contents of 352 specimens of *C. franciscorum* and 198

specimens of *P. macrodactylus* were examined. Percent frequency of occurrence of various food items was compared to derive an understanding of each one's relative importance in the diet of *C. franciscorum* and *P. macrodactylus*.

To determine whether certain lengths of *N. mercedis* individuals were being selected over others, Ivlev's (1961) index of electivity (E) was employed: $E = (F - A) \div (F + A)$, where F is the proportion in the foregut of a given length of specimens of *N. mercedis* relative to all lengths consumed, and A is the proportion available of the same length as F relative to all lengths available. E ranges from -1.0 to +1.0, with strong electivity of a length indicated in values near +1.0, and strong electivity against indicated in values near -1.0.

C. franciscorum and *P. macrodactylus* predation on *N. mercedis* populations was measured in each diel cycle in terms of the percent loss per m^3 per day of individuals and biomass (%I and %B below). Percentages were calculated as follows:

$$I = 3CN \quad \%I = 100I/(D_I + I)$$

$$B = \sum_{l=2}^m (I P_l W_l) \quad \%B = 100B/(D_B + B)$$

where I and B are respectively, the number of individuals and the dry weight (mg) of *N. mercedis* consumed per m^3 per day by specimens of *C. franciscorum* or *P. macrodactylus*, C represents the number of specimens of *C. franciscorum* or *P. macrodactylus* per m^3 , N represents the mean number of specimens of *N. mercedis* per *C. franciscorum* or *P. macrodactylus* foregut over a diel cycle, and 3 represents the number of foregut clearances per day by either predator. P_l = the proportion of each total length class l relative to the number of specimens of *N. mercedis* in all length classes for a diel cycle, with m being the longest class. W_l is the dry weight for each length class of specimens of *N. mercedis* determined from the equation given in Table I. D_I and D_B are respectively, the mean number of individuals and biomass (mg dry wt) of *N. mercedis* per m^3 for a diel cycle. The foregut clearance value of 3 was obtained from unpublished preliminary experiments conducted here by B. Louks on specimens of *C. franciscorum*, which indicated a 6-hr foregut clearance time, and from the estimate by Wilcox and Jeffries (1974) of 6 to 12 hrs for *C. septemspinosus*. We used an 8-hr clearance time as

TABLE I

Equations for length vs. dry weight equations for specimens of *Crangon franciscorum*, *Palaemon macrodactylus*, and *Neomysis mercedis*. $100 R^2$ is the coefficient of determination in percent (variation accounted for by the equation), n is the number of samples, W is the dry weight (mg), l is the total length (mm), and CL is carapace length (mm).

Species	n	Equation	100 R^2
<i>C. franciscorum</i>	53	$W_{CL} = 0.8789 (CL)^{2.474}$	92
<i>P. macrodactylus</i>	31	$W_{CL} = 0.6390 (CL)^{2.632}$	95
<i>N. mercedis</i>	176	$W_l = 0.01 \cdot 0.6145 (l)^{2.710}$	97

an approximation for *C. franciscorum* and applied it arbitrarily to *P. macrodactylus*. Statistical comparisons followed procedures of Steel and Torrie (1960).

RESULTS

Table I gives equations for estimating dry weight from length for specimens of *Crangon franciscorum*, *Palaemon macrodactylus*, and *Neomysis mercedis*. All have coefficients of determination ($100R^2$), or the percent of variation in weight accounted for by the equation, that are $\geq 92\%$.

The mean dry weight (mg) per m^3 for each diel cycle differed between species (Fig. 1). The biomass of the *N. mercedis* population was greater than the biomass of populations of *C. franciscorum* and *P. macrodactylus* in February and May. In July, the biomass of the population of *N. mercedis* was greater than the population biomass of *P. macrodactylus* but equaled the population biomass of

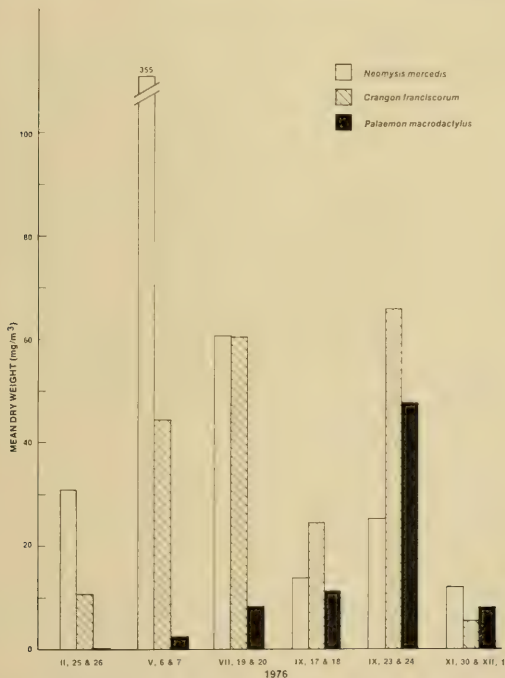


FIGURE 1. The mean dry weight (mg)/ m^3 of specimens of *Neomysis mercedis*, *Crangon franciscorum*, and *Palaemon macrodactylus* over diel cycles during 1976.

C. franciscorum. In September, the population biomass of *N. mercedis* was less than that for the population of *C. franciscorum* and was approximated and exceeded by the *P. macrodactylus* population. In July through late November diel samplings,

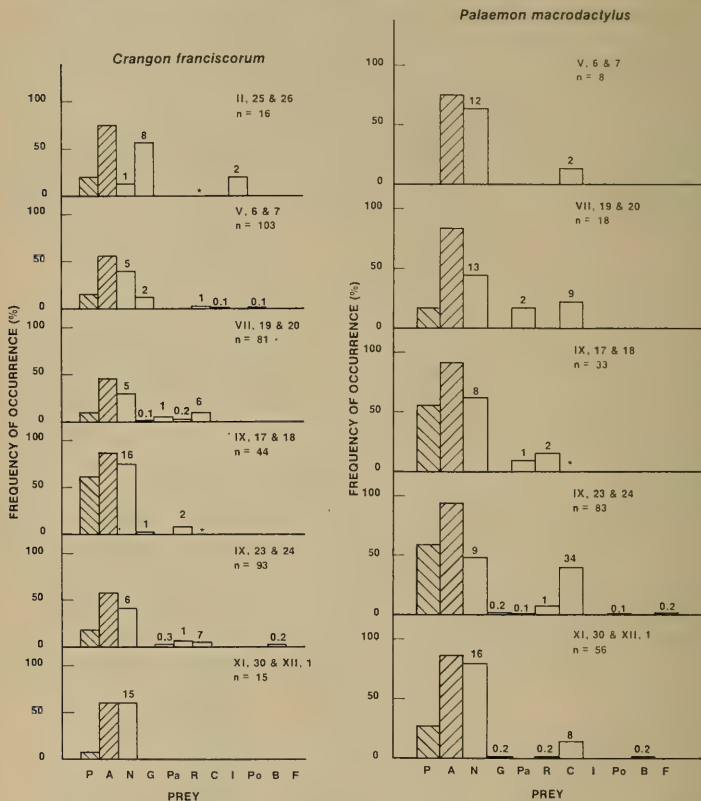


FIGURE 2. The frequency of occurrence of various items in the foreguts of specimens of *Crangon franciscorum* and *Palaemon macrodactylus* during 1976. Categories are plant fragments (P), animals or animal fragments (A), specimens of *Ncomysis mercedis* (N), gammarid amphipods (G), larvae of *Palaemon macrodactylus* (Pa) and the mud crab *Rhithropanopeus harrisi* (R), copepods (C), insects (I), polychaetes (Po), bivalves (B), and fish larvae (F). A star indicates that copepods were not counted. The number over a bar is the average number of that prey item per 10 foreguts over the diel cycle. The single *Palaemon macrodactylus* in February (II, 25 & 26) was excluded.

N. mercedis biomass was equaled or exceeded by the sum of *C. franciscorum* plus *P. macrodactylus* biomass.

Each species peaked in biomass at different times (Fig. 1). The biomass of *N. mercedis* was greatest in the May evaluation. *C. franciscorum* was most abundant in the May through September diel samplings, while *P. macrodactylus* biomass steadily climbed to a maximum in the late September diel cycle.

Foregut contents of *C. franciscorum* and *P. macrodactylus* indicate that those two species are mainly predacious. The frequency of occurrence of animals or animal fragments (A) always exceeded that of plant fragments (P) by at least

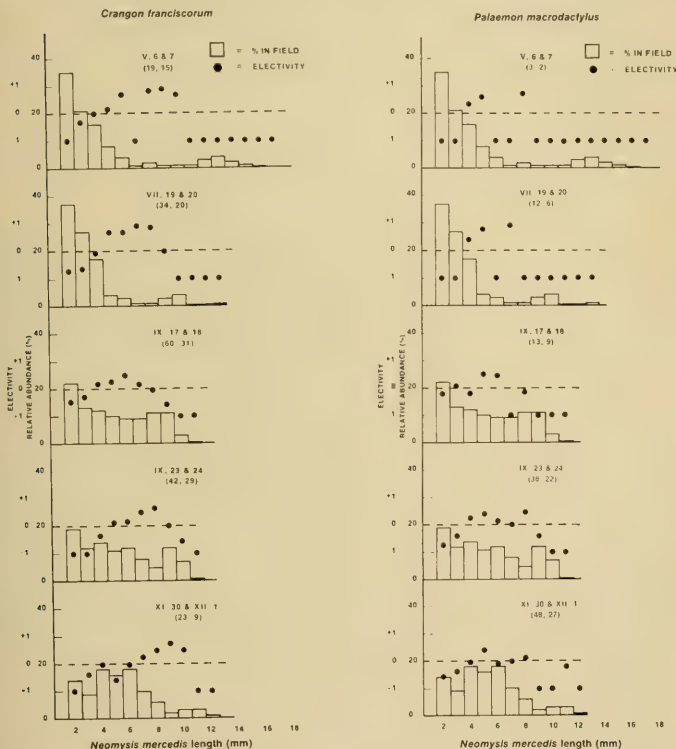


FIGURE 3. Size electivity (E) of *Crangon franciscorum* and *Palaemon macrodactylus* for specimens of *Neomysis mercedis* during diel cycles in 1976, and the relative abundance (%) of the total *N. mercedis* population in each length class.

25% (Fig. 2). Plant fragments were always small, and algae were rarely observed. The frequency of occurrence of animal fragments ranged from 46 to 86% in *C. franciscorum* and from 75 to 93% in *P. macrodactylus*. The frequency of plant fragments ranged from 7 to 61% in *C. franciscorum*, and from 0 to 59% in *P. macrodactylus*.

A variety of prey items were found in the foreguts of *C. franciscorum* and *P. macrodactylus*. Prey of both species included *N. mercedis*, gammarid amphipods including *Corophium* spp., larvae of *P. macrodactylus* and the mud crab *Rhithropanopeus harrisi*, copepods, polychaetes, bivalves, and fish larvae (closely resembling larval anchovies, *Engraulis mordax*). *N. mercedis* was the most frequently occurring item in both predators, found in 12 to 75% (February and September, respectively) of the *C. franciscorum* specimens and 44 to 79% (July and late November, respectively) of the *P. macrodactylus* specimens. The only difference in prey between the predators was that insect fragments were found only in specimens of *C. franciscorum*.

The prey items most abundant in the foreguts of specimens of *C. franciscorum* and *P. macrodactylus* were *N. mercedis* and copepods (Fig. 2). The number of specimens of *N. mercedis* per ten foreguts ranged from 1 to 16 for *C. franciscorum*, and from 8 to 16 for *P. macrodactylus*. Copepods per ten foreguts ranged from 0 to 6 for *C. franciscorum*, and from 2 to 34 for *P. macrodactylus*.

C. franciscorum selected *N. mercedis* specimens of intermediate sizes (Fig. 3). Consistently, mysids in the range of 6 to 9 mm long had the greatest positive electivity values. Selection was strongly against the most abundant smaller sizes, as well as the longer forms, with some electivity values of -1.0 . Larger individuals of *C. franciscorum* contained larger specimens of *N. mercedis* (Fig. 4). The mean length of specimens of *N. mercedis* consumed increased significantly from 4 to 8 mm as the carapace length of specimens of *C. franciscorum* increased from 5 to 10 mm. The mean length of *N. mercedis* specimens was only 5 mm for 11 mm specimens of *C. franciscorum*.

P. macrodactylus tended to select intermediate size specimens of *N. mercedis* (Fig. 3), as evident from the higher positive electivity values for 4 to 8 mm mysids. As with *C. franciscorum*, *P. macrodactylus* selected against both the most abundant shorter lengths and the longer specimens of *N. mercedis*, which equaled or surpassed the abundance of intermediate lengths in May through late September diel samplings. Larger specimens of *P. macrodactylus* consumed larger specimens of *N. mercedis* (Fig. 4). This relationship was consistent over all carapace lengths evaluated. The average length of specimens of *N. mercedis* consumed increased significantly (from 5 to 8 mm) with an increase in *P. macrodactylus* carapace length from 5 to 12 mm.

Variation in the number of specimens of *N. mercedis* per *C. franciscorum* foregut between depths was not significant (F-test, $\alpha 0.05$) either during the day or night except for day data in May. Here, the mean number of specimens of *N. mercedis* per *C. franciscorum* foregut was greater at 9 m (0.76) than at 5 m (0.27), with no specimens of *C. franciscorum* present at 1 m. Since depth made little difference, all depths were combined in comparisons between day and night feeding data.

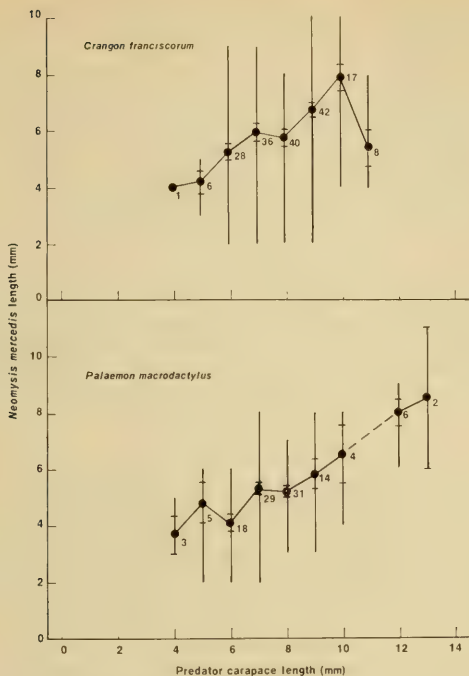


FIGURE 4. Range (vertical bar), mean (closed circle), and $\pm$ standard error (horizontal bars on range lines) of *Neomysis mercedis* lengths found in each carapace length (mm) class of *Crangon franciscorum* and *Palaemon macrodactylus*.

The number of specimens of *N. mercedis* per *C. franciscorum* foregut at night equaled or exceeded the day level. In the first four diel samplings, F-tests for variation in mysids per *C. franciscorum* foregut between day and night were not significant ($\alpha 0.05$) for means which ranged from 0.20 to 1.56 and 0.00 to 1.68 for day and night data, respectively. The late September night mean of 0.75 specimens of *N. mercedis* per *C. franciscorum* foregut was significantly ($\alpha 0.05$) greater than the day mean of 0.33. Similarly, the mean number of specimens of *N. mercedis* per *C. franciscorum* foregut in late November was significantly ($\alpha 0.01$) greater at night (3.00) than during the day (0.56).

Variation in the number of *N. mercedis* specimens per *P. macrodactylus* foregut between depths was significant (F-tests, $\alpha 0.05$) at night in late September and late November (specimens of *P. macrodactylus* were caught at only one depth

(9 m) during the day). The mean number of mysids per foregut at 1, 5, and 9 m at night in late September and late November were respectively, 0.00, 0.58, 1.07, and 0.33, 1.23, 1.83.

There was no significant ($\alpha 0.05$) difference in the number of specimens of *N. mercedis* per *P. macrodactylus* foregut between day and night samples in May, mid and late September, and late November. (F-tests for February and July data were not possible.) In these cases, mean mysids per *P. macrodactylus* ranged from 0.83 to 1.60 for day and 0.79 to 2.50 for night.

C. franciscorum and *P. macrodactylus* consumed *N. mercedis* before being caught and concentrated in the nets. The longest tows were near five minutes duration, and it is assumed that this period is insufficient for either predator to capture, macerate, swallow, and digest *N. mercedis* to the point that the lengths would be unmeasurable. Of the 144 specimens of *C. franciscorum* and 117 specimens of *P. macrodactylus* containing *N. mercedis* specimens respectively, 39% and 65% contained specimens of *N. mercedis* digested beyond the point at which the form of the carapace and abdomen fragments were sufficient for length determination.

N. mercedis standing crop consumed daily varied seasonally, with the extent of predation varying similarly among *C. franciscorum* and *P. macrodactylus*

TABLE II

Losses of Neomysis mercedis standing crop, in terms of individuals and biomass, to predation by populations of Crangon franciscorum and Palaemon macrodactylus during each diel sampling. Percentages (% L) are for loss relative to standing crop plus loss. $\bar{x}$ N.m./100 m³ is the mean number of Neomysis mercedis specimens per 100 m³. N_c and N_P are the individuals or biomass of Neomysis mercedis consumed per 100 m³ per day by populations of Crangon franciscorum (N_c) and Palaemon macrodactylus (N_P), and N_T is the total consumption by both predator populations. % BCC and % BPC are percents of the mean dry weight (mg)/m³ of Crangon franciscorum (BCC) and Palaemon macrodactylus (BPC) populations that each predator population consumed as Neomysis mercedis for each diel period.

Dates of diel samplings in 1976 (mo., days)	$\bar{x}$ N.m./100 m ³	N_c	% L	% BCC	N_P	% L	% BPC	N_T	%
individuals									
Feb. 25, 26	1,900	2	0.1	—	0	0	—	2	0.1
May 6, 7	38,400	33	0.1	—	5	0.01	—	38	0.1
Jul. 19, 20	14,600	77	0.5	—	15	0.1	—	92	0.6
Sep. 17, 18	1,600	105	6.2	—	34	2.1	—	139	8.0
Sep. 23, 24	2,500	103	4.0	—	127	4.8	—	230	8.4
Nov. 30 and Dec. 1	1,500	12	0.8	—	47	3.0	—	59	3.8
biomass									
Feb. 25, 26	3,100	—	—	—	0	0	—	—	—
May 6, 7	35,500	39	0.1	0.9	4	0.01	1.5	43	0.1
Jul. 19, 20	6,100	65	1.1	1.1	9	0.1	1.1	74	1.2
Sep. 17, 18	1,400	76	5.1	3.1	17	1.2	1.5	93	6.2
Sep. 23, 24	2,500	131	5.0	2.0	97	3.7	2.0	228	8.4
Nov. 30 and Dec. 1	1,200	16	1.3	2.9	33	2.7	4.1	49	3.9

(Table II). The percent of *N. mercedis* standing crop consumed was minimal during diel periods in February, May, and July. Mysid standing crop consumed by both predators was greatest in the September diel cycles. The decrease in *N. mercedis* standing crop due to *C. franciscorum* and *P. macrodactylus* predation combined were greatest during the late September diel cycle, with 8.4% of both the individuals (230) and dry weight (228 mg) of *N. mercedis* consumed per 100 m³ per day. *C. franciscorum* consumed more *N. mercedis* individuals than *P. macrodactylus* in all diel samplings except those in late September and late November. Also, except for the late November diel cycle, *C. franciscorum* consumed more *N. mercedis* biomass than did *P. macrodactylus*.

DISCUSSION

C. franciscorum and *P. macrodactylus* are mainly carnivorous and *N. mercedis* is the major identifiable prey for both species. The carnivore role probably exceeds the detritivore role more than is indicated by the frequency-of-occurrence results (Fig. 2). Plant fragments observed in the foreguts of both predators were filamentous strands, small in size relative to other material, and often could have originated in the foreguts of consumed prey.

Mysids were the major prey item mainly because they occurred most frequently in foreguts, except for *C. franciscorum* in February. Also, with the additional exception in *P. macrodactylus* in late September, there were more *N. mercedis* specimens per foregut than any other prey. *N. mercedis* individuals were the largest size prey consumed except for the infrequent polychaetes. Copepods were abundant in foreguts of *P. macrodactylus* specimens; however, since they are small they contribute less biomass than mysids.

C. franciscorum and *P. macrodactylus* are affecting the mysid population structure by preying mainly on intermediate sized (4 to 7 mm) specimens of *N. mercedis*. The more abundant smaller sizes may be too difficult to locate, while the larger sizes, which are often more abundant than intermediate sizes (Fig. 3), may be too quick to capture. By not preying mainly on larger sizes, *N. mercedis* recruitment and subsequent availability are enhanced because female mysids begin to become gravid when they are 7 mm long (Heubach, 1969).

C. franciscorum and *P. macrodactylus* are unable to exploit the greater daytime abundance of their major food resource near the bottom. The daytime average of *N. mercedis* specimens per predator foregut never exceeded the average for nighttime, when mysids are more dispersed. This may be a result of having to counter vertical transport in persistent turbulent flows during the day, perhaps to avoid being visible prey for fish. Opposing vertical transport may mean repeatedly swimming back toward the bottom and concurrent reduction in predation efficiency.

Nocturnal vertical migration has been hypothesized as a mechanism for avoiding visual predation (Zaret and Suffern, 1976), and sightless predation may be avoided in such migrations as well. Predation by individuals of *C. franciscorum* and *P. macrodactylus* is probably a sightless process since arthropod eyes are inferior in image formation (Goldsmith, 1973) and since individuals of both species prey at night and in darker regions during daytime. There is more time for predation at night since opposing vertical transport to avoid visibility is incon-

sequential. However, this sightless predation is avoided to some extent by *N. mercedis* through its dispersion in its nocturnal vertical migration.

Opposition of vertical transport during the day, and prey dispersion at night could keep the average number of mysids per foregut unchanged in most diel cycles. However, predation at night is more effective over some cycles for *C. franciscorum*. Also, another crangonid shrimp, *C. septemspinosa*, is indicated as feeding more at night from observations of greater volumes of stomach contents at night (Wilcox and Jeffries, 1974).

C. franciscorum and *P. macrodactylus* had their greatest impact on the standing crop of *N. mercedis* in September, and mysid recruitment was low in the following weeks, as indicated by the low abundance of 2 to 3 mm mysids in late November (Fig. 3). At a daily 8% decrease in *N. mercedis* standing crop, and with other factors unchanged in their effects on predation, only 1% of the September standing crop of *N. mercedis* would have been left by the time of the late November diel sampling. What probably prevented a portion of this decimation was the reduction in numbers of *C. franciscorum* and *P. macrodactylus* individuals, as indicated by the drop in their biomass (Fig. 1). *C. franciscorum* has been observed to move to greater salinities during cooler periods (Israel, 1936; Krygier and Horton, 1975). *P. macrodactylus* probably emmigrated also, perhaps seaward and to slower, shallower channels and marshes, for it is adapted for walking and climbing and has been found in such habitats (Newman, 1963; Little, 1969).

The similarities between *C. franciscorum* and *P. macrodactylus* raise the question of interspecific competition, both between themselves and between either of them and fish. *C. franciscorum* and *P. macrodactylus* specimens of similar size, coexist, undergo nocturnal vertical migrations, and have similar prey. Both have their greatest impact on the standing crop of *N. mercedis* in September, when their standing crops are at intermediate to high levels. It is during September that there is the greatest chance of interspecific competition for food (*N. mercedis*) between *C. franciscorum* and *P. macrodactylus*. The biomass of *N. mercedis* consumed by each predator population relative to its own standing crop was not low in September in comparison with other months (Table I) indicating that food supply was not limiting. Competition between *C. franciscorum* or *P. macrodactylus* and fish is possible but most likely only to occur in the fall, when *N. mercedis* is in relatively low abundance, but when the caridean shrimps are abundant. Unfortunately, no information was available on the biomass, distribution, and feeding of the fishes present.

Interspecific competition between *C. franciscorum* and *P. macrodactylus* may increase in the future. *P. macrodactylus* is an exotic species, thought to have been introduced into the Sacramento-San Joaquin Estuary in the first half of this century (Newman, 1963), and the relationship between the two species may not yet have stabilized. *P. macrodactylus* may have an important advantage over *C. franciscorum* in the study area, because only *P. macrodactylus* reproduces there, whereas *C. franciscorum* migrates seaward to reproduce. Data are still insufficient to speculate on the extent to which *P. macrodactylus* may displace *C. franciscorum*.

Changes in environment relative to conditions at the time and place of this

study may modify indications in these results. Drought in California in 1976 led to an intrusion of salinity unusually far upstream. Also, *N. mercedis* abundance during the same period was below average (Orsi and Knutson, in preparation).

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SUMMARY

1. Feeding studies were conducted on the caridean shrimps *Crangon franciscorum* and *Palaeomon macrodactylus* of the Sacramento-San Joaquin Estuary, California. Foregut contents from 352 specimens of *C. franciscorum* and 198 specimens of *P. macrodactylus* collected over six diel cycles in 1976 were itemized.

2. *C. franciscorum* and *P. macrodactylus* are mainly carnivorous. Animal material always exceeded plant fragments in frequency of occurrence in foreguts, being respectively 46 to 86% and 7 to 61% in *C. franciscorum* and 74 to 92% and 0 to 59% in *P. macrodactylus*. The most frequent prey item identifiable in both predators was the mysid shrimp *Neomysis mercedis*. Both predators selected for *N. mercedis* of intermediate lengths (5 to 9 mm), and selected against those 2 mm or >10 mm long.

3. Day and night differences in the number of individuals of *N. mercedis* per foregut were significant only for *C. franciscorum*, and then only in late September and late November, with greater means at night in each case.

4. The percentages of *N. mercedis* standing crops consumed daily by *C. franciscorum* and *P. macrodactylus* respectively, ranged from 0.1 to 6.2% and 0.0 to 4.8% in terms of individuals, and from 0.1 to 5.1% and 0.0 to 3.7% for biomass. The percent of their respective population biomasses that specimens of *C. franciscorum* and *P. macrodactylus* respectively consumed daily as *N. mercedis* ranged from 1 to 3% and 1 to 4%. Similarities between these predators suggest the possibility of competition.

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IRON AND MANGANESE DEPOSITION IN THE PERIOSTRACA OF SEVERAL BIVALVE MOLLUSCS

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The presence of high levels of iron (Fe) and manganese (Mn) in the shells of freshwater and marine molluscs is a consistent observation in the literature. It has been reported that the shell of a freshwater lamellibranch in the genus *Anodonta* has Fe and Mn levels of 2600 and 560 ppm (0.26 and 0.056%, respectively). Seven marine lamellibranchs and four marine gastropods had Fe and Mn levels ranging between 4.5 and 1600 ppm and 1.1 and 18 ppm, respectively (Segar, Collins and Riley, 1971). It was not determined whether the Mn or Fe was concentrated in a particular region of the shell. In some cases Mn is found concentrated on the outer surface of shells. Allen (1960) examined the Mn present in the surface deposits on a variety of marine molluscs. For *Nucula sulcata*, Mn levels in surface deposits were reported to be 8.5 and 17.9%. In regions where *N. sulcata* was found, the bottom sediments contained Mn at 0.1%. Zavarzin (1964) reported that Mn is deposited on the shell of the marine mollusc *Macoma baltica* L. Both Allen and Zavarzin suggested that the process by which Mn is concentrated is biological and bacterial. Zavarzin concluded that Mn^{2+} exchanges into the $CaCO_3$ structure of the shell and microorganisms cause the oxidation of Mn^{2+} on the shell surface to manganese oxides. The bacterial oxidation of Mn^{2+} to oxides of higher oxidation states of Mn is well documented (Ehrlich, 1968; Branter, 1970; Buchanan and Gibbons, 1974).

The specific experiments described here are focused on the incorporation mechanism and on the chemical characteristics of the metals found in the shells of molluscs. The Mn and Fe levels in the shells of several freshwater and marine molluscs were examined by the electron microprobe technique and a variety of physical and chemical methods. It was found in the cases of the freshwater molluscs, *Anodonta californiensis* and *Unio novahollandae*, that Mn and Fe were concentrated locally at high levels (up to 13 and 40%, respectively) in their periostraca, while in the case of the marine mollusc, *Mytilus californianus*, Mn and Fe were not detectable ($< 0.05\%$) by electron microprobe methods in the periostracum or shell.

MATERIALS AND METHODS

Specimens of the freshwater mussel *Anodonta californiensis* were collected in irrigation canals 15 miles north of Sacramento, California, and specimens of another freshwater mussel, *Unio novahollandae*, were collected at the Mt. Esk Pocket on the Brisbane River west of Brisbane, Queensland, Australia. *A. californiensis* was found in mud and *U. novahollandae* in gravel or mud. In the case of *A. californiensis*, mud and water samples were collected. Specimens of the marine mussel *Mytilus californianus* were collected at Dillon Beach, California

from rocks washed by surf. The shells of the marine specimens were removed, washed in fresh, filtered sea water, and then rinsed in distilled water and dried in air. Depending on the experiment to be performed, the freshwater specimens were either stored in an aquarium with water from the collection site, or with distilled

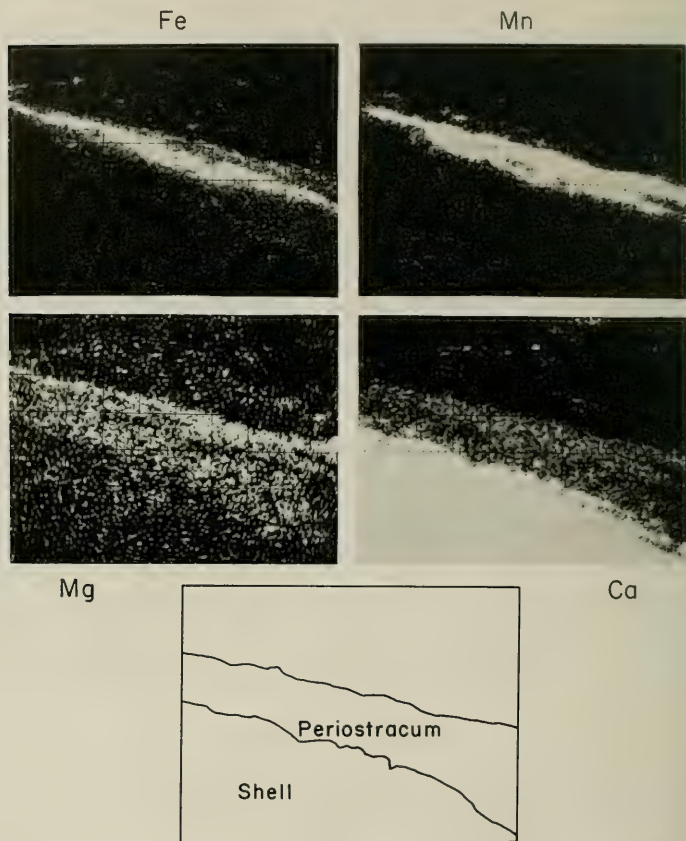


FIGURE 1. Scanning displays of Fe, Mn, Mg and Ca for a section of the shell of a specimen of *A. californiensis*, and a diagram of the limits of the periostracum and CaCO₃-containing region.

water, and the shell removed after an appropriate time; or the shells were removed directly on collection. The shells were rinsed in distilled water, and air dried.

The periostracum was removed from the shell by scraping or by application of a vacuum to the shell. The CaCO_3 -containing layer underlying the periostracum was removed with the periostracum, and for quantitative metal analyses the per cent periostracum in any sample was determined independently. The periostracum itself is not dissolved by acids such as HCl (Beedham, 1958), so the amount of CaCO_3 was determined by treatment with acid and measurements of the CO_2 evolved, and/or by measurement of the remaining periostracum.

Metal analyses for Mn and Fe were performed using standard, quantitative, colorimetric methods (for Mn oxidation to permanganate, and for Fe complexing with thiocyanate), and levels of Mn, Fe and Mg were determined by atomic absorption. Phosphate, PO_4^{3-} , analyses were done by a standard method (Lucena-Conde and Prat, 1957).

Local metal concentrations in the periostraca, as well as electron microprobe scanning displays for various metals, were carried out on sections of the shells using standard methods on an Applied Research Laboratory model EMX-SM electron microprobe. The metals or metal-doped CaCO_3 or $\text{Ca}_3(\text{PO}_4)_2$ were used as standard.

X-ray scattering patterns of the periostracum were determined using a Siemens X-Ray Diffractometer. Bacterial cultures grown from portions of the periostracum were prepared by standard methods and examined.

RESULTS

Figures 1, 2 and 3 show typical electron microprobe scanning displays for Fe, Mn, Mg and Ca from sections of the shells of the freshwater molluscs *Anodonta californiensis* and *Unio novahollandae*, and the marine species *Mytilus californianus*. The accompanying diagrams indicate the limits of the periostraca and shells. The metals Sr, Cu, Co, Ni and Zn were not detected by electron probe measurements and, therefore, are at levels less than 0.05%. No attempt was made to determine their presence by other analytical methods. The periostraca of the freshwater species showed high levels of Fe and Mn, but the periostraca of the marine species have Fe and Mn levels at less than 0.05%. Local Fe and Mn levels for *A. californiensis* and *U. novahollandae* periostraca range up to 5% (Fe) and 15% (Mn), and 13 and 40%, respectively.

The amounts of Fe, Mn and Mg in the entire periostracum were measured by techniques already discussed. There is some variation in the results, since electron probe measurements showed substantial variations in the amounts of these metals within the periostracum. Samples used for analysis contained several portions of periostracum taken from several shells. They also contained both the periostracum and parts of the CaCO_3 -containing layer underlying the periostracum. The per cent of periostracum in samples of *A. californiensis* ranged from 7 to 10%, and the appropriate number was applied to analyses performed. Analyses were carried out on samples containing periostracum and CaCO_3 layers, since electron probe measurements showed almost all of the metals to be in the periostracum. Analyses were run on periostraca of *A. californiensis* specimens that had been washed briefly

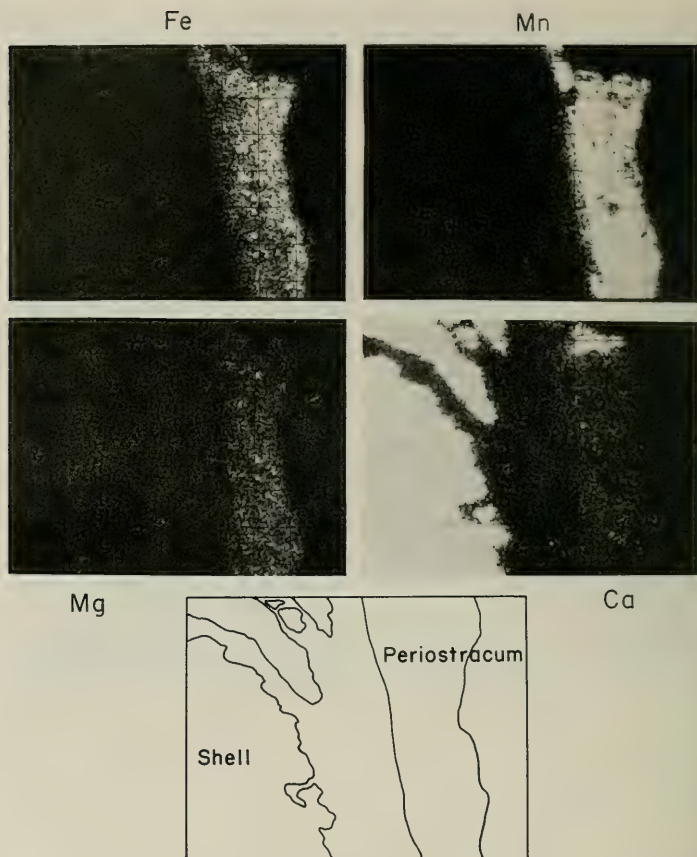


FIGURE 2. Scanning displays of Fe, Mn, Mg and Ca for a section of the shell of a specimen of *U. novahollandiae* and a diagram of the limits of the periostracum and CaCO_3 -containing region.

with distilled water after collection and put in an aquarium containing fresh water, where they remained for several days. Typical analyses for various metals were: (Fe) 0.6%, 1.3; (Mn) 2.6, 1.1; (Mg) 0.15, 0.11, and (Fe) 2.7; (Mn) 0.48, 0.37, 0.77. The percentages recorded for the two analyses are from different

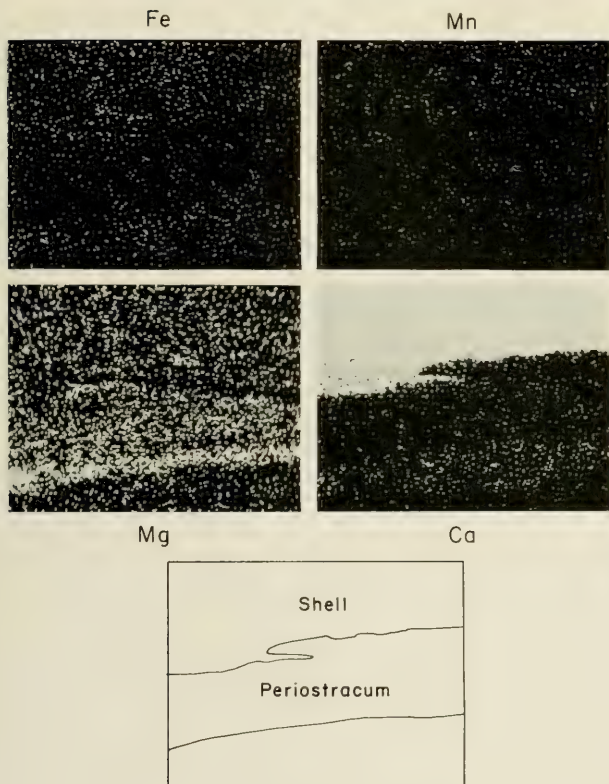


FIGURE 3. Scanning displays of Fe, Mn, Mg and Ca for a section of the shell of a specimen of *M. californianus* and a diagram of the limits of the periostracum and CaCO_3 -containing region.

samples. The lower Mn content in the second analysis may be a manifestation of the fact that manganese is slowly lost from specimens in an aquarium. In any case, these numbers represent approximate levels of Mn, Fe and Mg in the total periostracum of *A. californiensis*.

Figure 4 represents electron microprobe scanning displays for Ca, Mn and P for a shell section from a specimen of *A. californiensis*. There is an exact correspondence of the regions occupied by these elements. The density map for

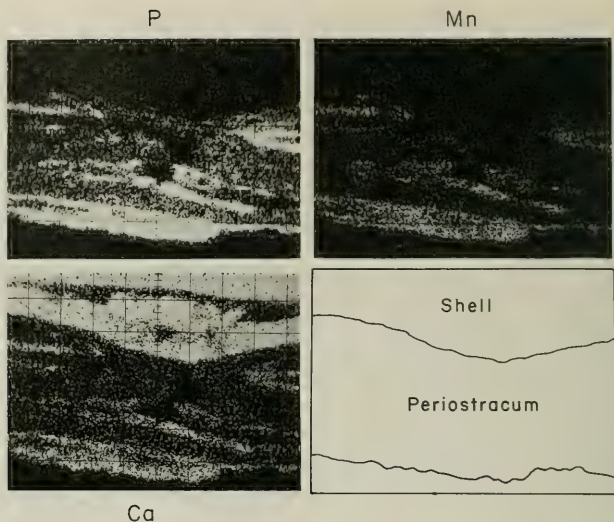


FIGURE 4. Scanning displays of P, Mn and Ca for a section of the shell of a specimen of *A. californiensis* and a diagram of the limits of the periostracum and CaCO₃-containing region.

Fe shows an approximate correspondence with the regions occupied by Ca, Mn and P, but Fe is at lower levels than Mn and is contained in other regions as well. Infrared spectra of periostraca contained bands characteristic of PO_4^{3-} , and PO_4^{3-} is found analytically (Lucena-Conde and Prat, 1957).

Table I contains data on the weight percentages of Ca, Mn and P at various points in the periostracum of *A. californiensis* as determined by electron microprobe analyses; and the quantities $(\text{Ca} + \text{Mn}/1.37)$, the weight per cent Ca that would occur if Mn was converted to Ca, and $(\text{Ca} + \text{Mn}/1.37)/\text{P}$. The latter two quantities will be used later on in this paper.

The Ca, Mn and P contents of the periostracum of *U. novahollandae* were measured. The P content of the periostracum was measured at 8 points and ranged between 0.1 and 0.6%, the Ca content between 1.0 and 4.8%, and the Mn content between 8.5 and 39.0%. The ratios of $(\text{Ca} + \text{Mn}/1.37)/\text{P}$ were considerably larger than those of *A. californiensis* because of the extremely large Mn levels, but in no case were they less than the value for $\text{Ca}_3(\text{PO}_4)_2$ itself. Phosphorus was not detectable in the CaCO₃-containing regions of the shells of either species.

In the case of *A. californiensis*, the mud and water surrounding the specimens were analyzed. The Mn and Fe contents of the mud based on dry weight were 0.25 ± 0.02 (4 determinations) and $3.29 \pm 0.04\%$ (3 determinations), respectively.

TABLE I

Weight per cents Ca, Mn and P and derived quantities for the periostracum of specimens of *A. californiensis*.

Ca	Mn	Ca + $\frac{\text{Mn}}{1.37}$	P	Ca + $\frac{\text{Mn}}{1.37}$ P
1.16	0.06	1.2	0.62	1.9
3.8	0.54	4.2	2.5	1.7
4.8	0.77	5.4	2.8	1.9
6.1	1.0	6.8	3.7	1.8
6.5	1.3	7.5	3.9	1.9
7.7	1.9	9.1	6.2	1.5
8.5	1.4	9.5	4.3	2.2
8.8	1.5	9.9	5.0	2.0
13.0	2.9	15.1	7.9	1.9
20.5	4.5	23.8	12.0	2.0

Average = 1.9 ± 0.1

The water had concentrations of Mn and Fe of less than 10^{-8} and 10^{-6} M, respectively. The substrate in which *U. novahollandae* was found was not analyzed, but consisted of areas of mud and gravel.

Electron spin resonance (ESR) measurements were carried out on the periostracum to learn something about the chemistry (oxidation states and environments) of the metals in the periostracum. ESR signals are observed when molecules having unpaired electrons are placed in a magnetic field. Figure 5a shows the ESR spectrum of a fresh sample of periostracum of *A. californiensis*, and Figure 5b is an expansion of the center portion of Figure 5a. A dominant feature of Fig. 5a is a six-line spectrum, which is characteristic of dilute samples of Mn^{2+} . The ESR spectrum of dilute Mn^{2+} in an octahedral field has six sets of a five-line spectrum, in which one of the five lines may be dominant. The basic features of the spectrum in Figure 5b can be simulated by superimposing two Mn^{2+} in CaCO_3 spectra in the ratio 0.64 to 1 separated by 29 gauss. Manganese (+2) from the salt MnCl_2 was coprecipitated with CaCO_3 for these experiments. ESR spectra of Mn^{2+} in MgO (Wertz and Bolton, 1972) and in barnacle shells (Blanchard and Chasteen, 1976) have been recorded in the literature, and have the same characteristics as Mn^{2+} in CaCO_3 . Therefore, it is clear that Mn^{2+} is present in two sites in which it is dilute. These are not sites associated with the Ca-P-rich regions, as was shown by experiments described further on in this paper. Potential contributors to the broadened portion of the spectrum shown in Figure 5a could be concentrated Mn^{2+} , where exchange broadening occurs; Mn^{4+} ; high spin Fe^{2+} ; and Fe^{3+} . Manganese (2+) at the levels found in the enriched Ca-P-containing regions was coprecipitated with $\text{Ca}_3(\text{PO}_4)_2$. The EPR spectrum was identical to that of the broad band to the right of 3340 gauss in the central region of Figure 5a. Manganese dioxide, MnO_2 , in glycine, but not Fe^{3+} or Fe^{2+} compounds, yielded an ESR spectrum which with the spectrum of Mn^{2+} in $\text{Ca}_3(\text{PO}_4)_2$ accounts for that portion of the broad band to the left of 3340 gauss. It seems reasonable that at least Mn^{2+} and

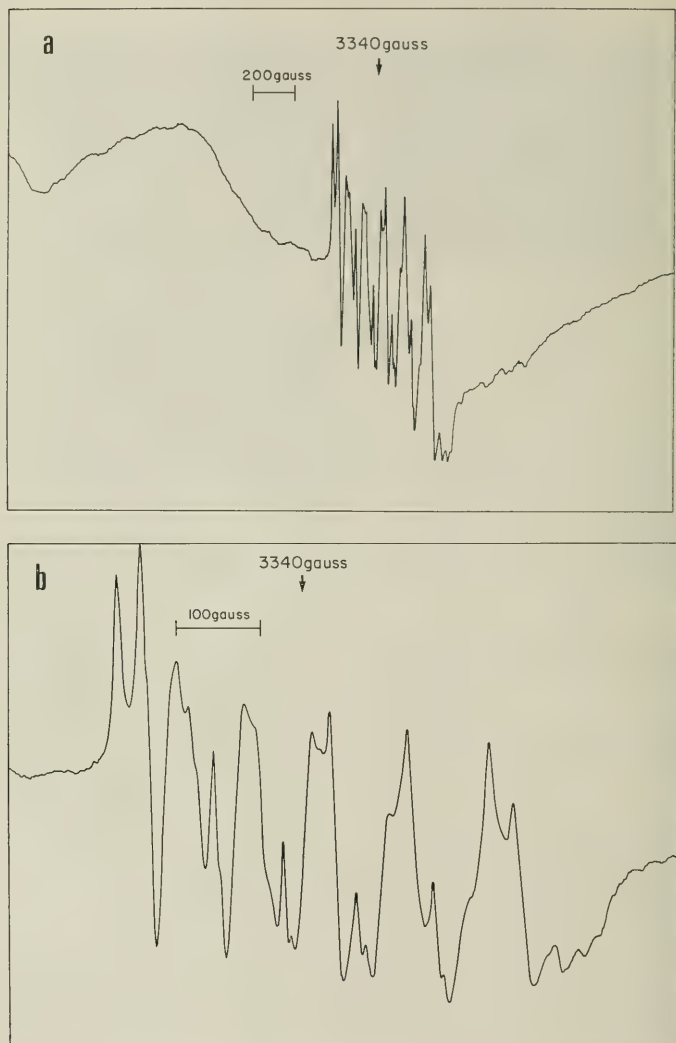


FIGURE 5. Part a shows the ESR spectrum of the periostracum of a specimen of *A. californiensis*. Part b shows the expansion of the central portion of the ESR spectrum.

Mn²⁺ (as MnO₂) contribute to the broad ESR spectrum. The ESR spectrum was taken of the mud in which the specimens were contained. The broad features of this spectrum are the same, but there is no sharp six-line Mn²⁺ spectrum. The ESR spectrum of the periostracum of *U. novahollandae* shows the same features as that of *A. californiensis*, including the multiple-line spectrum of Mn²⁺, but the entire spectrum is more intense, which is consistent with the higher levels of Fe and Mn found in the periostracum of the former.

Several chemical experiments were carried out on the metal-containing periostracum. It was noted that the periostracum of specimens of *A. californiensis*, which were left in an aquarium for a week, lost manganese. Three treatments at room temperature (50% acetic acid for 3 hr, or 0.1 M HCl for 30 min., or 0.1 M EDTA at pH = 11 for several hours) remove Mn but not Fe from the periostracum, as evidenced by analyses for Fe and Mn in the solutions and by disappearance of the dilute Mn²⁺ bands from the EPR spectrum. The addition of an aqueous solution of MnCl₂ to periostracum treated with 50% acetic acid for 3 hr or 0.1 M HCl for 30 min results in the uptake of the metal. In a typical experiment, 0.0271 g of decalcified periostracum which had been treated to remove Mn²⁺ was mixed into 0.10 liter of 0.10 M MnCl₂. Removal of the periostracum from the Mn-containing solution and retreatment with acetic acid indicated 1.2% manganese (by weight) of the periostracum had been taken up. The sharp ESR spectrum of Mn²⁺ did not return, indicating an irreversible change on treatment of the periostracum with acetic acid or HCl and/or removal of Mn²⁺, or a lack of generation of Mn²⁺ in magnetically dilute sites. Iron can be removed from the periostracum by long term contact of the periostracum with 0.1 M EDTA at pH < 8 or 6 N HCl for 2 hr, and tests with a variety of reagents under anaerobic conditions indicate the oxidation state to be +3.

The idea of bacterial involvement in the deposition of Mn was investigated to the extent that cultures were grown from scrapings from the periostracal surface of specimens of *A. californiensis*. No bacteria known to be involved in Mn²⁺ oxidation-reduction processes were observed (Ehrlich, 1968; Buchanan and Gibbons, 1974). The possibility exists that the animal takes up Fe and Mn, and then the metals and periostracum are produced at the appropriate mantle cells. Sections were taken of the mantle of specimens of *A. californiensis* and the cellular structure responsible for the periostracum (Wilbur, 1976) was examined by electron microprobe. No Fe or Mn was found.

DISCUSSION

In the electron microprobe scanning displays shown for *Anodonta californiensis*, and to a lesser extent *Unio novahollandae* (Figs. 1 and 2), the metals are located towards the outer surface of the periostracum. In the case of *U. novahollandae* the Mn gradient becomes apparent when the sensitivity of the measurement is reduced. The regions of the periostracum occupied by Fe and Mn are generally the same, but as can be seen in both figures, there is not an exact superposition and in some cases Fe is found independently. The Mg content of the periostracum is somewhat higher than that of the CaCO₃-containing layer adjacent to the periostracum. There is clearly Ca in the periostracum. The fact that the metals tend to be localized towards the outer surface of the periostracum suggests either that uptake occurs from the surrounding sediment or water, or the metals are

incorporated preferentially in certain layers of the periostracum produced by the animal. It appears that bivalves initially produce the outer periostracal layer, and that additional layers form on the inside of this layer (Clark, 1976).

The correspondence of Ca, Mn and P in the periostracum of *A. californiensis* (Fig. 4) and the detection of PO_4^{3-} analytically and by infrared spectrum suggests that Mn has been incorporated into a $\text{Ca}_3(\text{PO}_4)_2$ structure. To check whether $\text{Ca}_3(\text{PO}_4)_2$ with Mn^{2+} inclusion was present, the amounts of Ca, Mn and P in various regions of the periostracum were determined by electron microprobe measurements (Table I) and expressed as the ratio $(\text{Ca} + \text{Mn } 1.37)/\text{P}$. The points taken ranged from Ca, Mn and P values in per cent by weight of 20.5, 4.5 and 12.0 to 1.16, 0.06 and 0.62, respectively. The quantity Mn 1.37 corrects the Mn to Ca, and if Mn alone replaces Ca in $\text{Ca}_3(\text{PO}_4)_2$, the ratio $(\text{Ca} + \text{Mn } 1.37)/\text{P}$ should be that of $\text{Ca}_3(\text{PO}_4)_2$ itself. The average of 10 point measurements was 1.9 ± 0.1 . The ratio Ca, P in $\text{Ca}_3(\text{PO}_4)_2$ was 1.94. At the levels of Fe and Mg in the periostracum, correction of these elements to Ca, as was done for Mn, raised the theoretical Ca/P ratio by no more than 0.2 units. Since the per cents by weight of Ca and P in $\text{Ca}_3(\text{PO}_4)_2$ were 37.7 and 19.5, these values indicated $\text{Ca}_3(\text{PO}_4)_2$ was present but was diluted in a matrix of some other compound. It seems likely that this matrix contains protein, which is known to make up the organic component of the periostracum (Beedham, 1958). X-ray scattering experiments for the periostracum indicated that the $\text{Ca}_3(\text{PO}_4)_2$ was amorphous.

Both *A. californiensis* and *U. novahollandae* have elevated levels of P in the periostracum compared to the CaCO_3 -containing shell, and high levels of Ca, Mn, and P occupy the same regions of the periostracum. In the case of *A. californiensis*, where the Mn level is low, the evidence supports Mn^{2+} inclusion in $\text{Ca}_3(\text{PO}_4)_2$. In *U. novahollandae*, where substantial Mn accumulation has occurred, the ratio $(\text{Ca} + \text{Mn}/1.37)/\text{P}$ exceeds that of Ca/P in $\text{Ca}_3(\text{PO}_4)_2$. This could be related to the irreversible oxidation of Mn^{2+} to Mn^{4+} (*vide infra*) or the adsorption of Ca^{2+} on manganese oxides. The uncorrected Ca/P ratio exceeds that of $\text{Ca}_3(\text{PO}_4)_2$ and it is assumed carbonate accounts for the remainder of the counter ion.

The ESR spectra which show dilute Mn^{2+} in two sites probably represent contributions from Mn^{2+} in the CaCO_3 -containing layer underlying the periostracum, which cannot be separated from the periostracum, and the Ca-P-deficient regions of the periostracum. It is clear that Mn^{2+} , at the concentrations observed in the Ca-P-rich regions of the periostracum, will have a broad ESR spectrum, and it appears that Mn^{2+} and Mn^{4+} are the contributors to the broad spectrum shown in Figure 5a.

The explanation for the concentration of Mn and, to a lesser degree, Fe, by the periostraca of *A. californiensis* and *U. novahollandae*, and not by *M. californianus* requires some information about the organic makeup of this layer. The periostracum of *M. californianus* has been examined (Hare, 1963), and found to contain high levels of the amino acids glycine and tyrosine. The periostraca of two related species in the genera *Anodonta* (*A. cygnea*) and *Mytilus* (*M. edulis*) have been shown to have similar amino acid contents, being high in glycine and tyrosine (Beedham, 1958). In principle, such proteinaceous material can provide good complexing sites for metal ions. The presence of Ca-P-rich areas in

the periostracum, presumably as amorphous calcium phosphate, provides a medium in which Mn^{2+} can exchange with Ca^{2+} . The ionic radii of Mn^{2+} and Ca^{2+} are 0.80 and 0.99 Å, respectively, and Mn^{2+} is known to replace Ca^{2+} in a number of biological systems (Williams, 1970). It should be noted that the Ca-P-rich areas contain a diluent. It seems very likely this diluent is proteinaceous material, which can also provide complexing sites. Thus, one explanation for the high levels of Mn found in the periostraca of the freshwater molluscs is that Mn^{2+} can be effectively complexed by the proteinaceous material and/or Ca-P-rich areas, presumably exchanging with Ca^{2+} in a protein-calcium-phosphate matrix in the latter. After such complexation or exchange, the Mn^{2+} can only oxidize or remain in its present state. Some of the Mn remains as Mn^{2+} , since it can be removed from the periostracum by equilibration with water free of Mn^{2+} . Oxidation to oxides of higher oxidation states of Mn (i.e., MnO_2) would provide a "sink" for Mn and can account for the large Mn concentrations found in certain parts of the periostracum. As noted previously, such an oxidation could be bacterial, or could direct inorganic oxidation (Hubred, 1975) by dioxygen (O_2), or possibly Fe^{3+} and then oxidation of the resulting Fe^{2+} to Fe^{3+} by O_2 . The persistent observation of the coexistence of Fe and Mn together in the periostracum is interesting. It should be noted that the dominant metals present in manganese nodules are also Fe and Mn, and that the Mn content normally exceeds that of Fe, as it does in these periostraca. It has been suggested (Hubred, 1975) that an iron coated nucleus is present in nodules which catalyzes the oxidation of Mn^{2+} to manganese oxides, which are themselves catalysts for the process. Such a process could contribute to the accumulation of Mn and Fe in the periostracum, and their general occupation of the same regions.

It is unlikely that the Mn and Fe are being concentrated directly from the water, since the levels of the metals observed in the water at the time of collection are very low, and Mn^{2+} is slowly lost to water containing low Mn and Fe concentrations. This does not preclude the possibility that the Mn and Fe concentrations are high at specific times, but a much more likely possibility is that the source of the Mn and Fe is the mud in which the molluscs are imbedded. The levels found in the muds are high: 0.25% dry weight for Mn and 3.3% for Fe in the case of *A. californiensis*, but these metals would have to be concentrated by the mechanisms previously discussed to reach the local concentrations observed in the periostracum. It is not puzzling that periostraca of *M. californianus* contain no Mn or Fe, since the specimens were not in the proximity of muds or sands containing reasonable levels of the metals, but were on exposed rocks washed by surf. Also, sea water has a substantially higher level of Mg^{2+} than fresh water and Mg^{2+} can compete with Mn^{2+} for complexing sites (Williams, 1970). Both Mg^{2+} and Mn^{2+} are spherically symmetrical and thus have no specific geometrical requirements, and their ionic radii are 0.65 and 0.80 Å, respectively.

The potential influences of these concentrated metals on the mollusc and its environment are twofold. The high concentrations of manganese and iron (and potentially Ca) provide a defensive buffer against acidic attack on the periostracum of the mollusc itself. Although the periostracum is fairly resistant to acid (Beedham, 1958), it seems logical that these metals would be preferentially attacked

by acid. The presence of manganese and, to a lesser extent, iron associated with phosphate and protein, produces a condition which can maximize their nutritive availability to microorganisms.

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Examinations of bacterial cultures were performed by various members of the Department of Bacteriology, University of California, Davis. Sections of the mantle of specimens of *A. californiensis* were prepared for electron microprobe study by the Facility for Advanced Instrumentation, University of California, Davis. Samples of *U. novahollandae* were collected by Mr. O. Kelly, Department of Zoology, University of Queensland, Brisbane, Australia.

SUMMARY

The iron (Fe) and manganese (Mn) contents of the periostraca of specimens of the freshwater molluscs *Anodonta californiensis* and *Unio novahollandae*, and marine mollusc *Mytilus californianus* were determined. The freshwater molluscs concentrate Fe and Mn. The manganese being concentrated in regions high in Ca and P, which regions appear to be amorphous $\text{Ca}_3(\text{PO}_4)_2$ in a proteinaceous matrix. Iron appears in these Ca-P regions but also occurs elsewhere in the periostracum. Electron spin resonance (ESR) measurements indicate the oxidation states of the Mn to be +2 and +4, while chemical experiments suggest Fe is present in the +3 oxidation state. The levels of Mn and Fe in the periostracum can be several per cent. Local concentrations, determined by electron microprobe measurement, are as high as 15% and 5%, respectively for *A. californiensis*, and 40 and 13%, respectively for *U. novahollandae*. It is postulated that the Mn and Fe are removed directly from muds rich in these metals. Iron and Mn levels in the marine mollusc *M. californianus* do not locally exceed 0.05%.

Manganese and iron in the periostracum of a mollusc can act as a defensive buffer against degradation from acidic conditions. The concentration of these metals, in the presence of phosphate and protein, produces a condition which can maximize nutritive value to microorganisms.

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ANTENNULAR SENSILLA OF THE BRINE SHRIMP, *ARTEMIA SALINA*

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Cuticular sensilla of arthropods have been extensively studied in members of the class Insecta, and combined ultrastructural, physiological, and behavioral data have contributed greatly to our understanding of the functions and biological significance of such sensory structures (McIver, 1975; Slifer, 1970). By comparison, similar sensilla of members of the class Crustacea have received much less attention, and the majority of recent accounts have dealt solely with higher crustaceans, such as decapods (*e.g.*, Ball and Cowan, 1977; Snow, 1974; Tazaki, 1977; Vedel and Clarac, 1976). The present paper describes cuticular sensilla found on the antennules of the brine shrimp *Artemia salina*, a primitive crustacean belonging to the subclass Branchiopoda. Two distinct types of adult antennular sense organs are characterized by means of scanning and transmission electron microscopy. On the basis of the morphological findings, together with results from dye-penetration experiments, possible functions of the sensilla are discussed. Since the antennules of the naupliar larva of *Artemia* also bear setae that may be sensory in function, these larval structures were studied as well. Their external morphology is compared to that of the two types of sensilla found on antennules of adult brine shrimp.

MATERIALS AND METHODS

Source of animals

Specimens of live adult *Artemia salina* (L.) were obtained from a commercial supplier (Living World, San Francisco Bay Brand, Newark, California) or from private cultures. To the best of our knowledge, all brine shrimp were from the San Francisco Bay area or had been cultured from eggs collected there. Live nauplii were hatched from brine shrimp eggs obtained commercially (Living World Hatch Mix) and were fixed no later than 30 hr after hatching.

Scanning electron microscopy (SEM)

A variety of fixation procedures was employed to preserve adult brine shrimp. The following three methods were most often used and yielded favorable specimens for SEM:

Method 1. Whole adult shrimp were fixed in 10% acrolein in 0.1 M sodium cacodylate for one hr at room temperature and then washed and stored in cold 0.1 M sodium cacodylate for periods ranging from three days to one month. Prior to osmication, the head of each animal was cut off with iridectomy scissors. The

heads were post-fixed in 2% osmium tetroxide in 0.1 M sodium cacodylate for 1.5 hr at room temperature and rinsed quickly six times in water. Further osmication of the tissue was done by means of a thiocarbonylhydrazide (TCH) technique modified from that of Malick, Wilson, and Stetson (1975). The specimens were subjected to the following sequential treatments: immersion in fresh, saturated, aqueous TCH for 30 min; six rinses in water; immersion in aqueous 2% osmium tetroxide for 2 to 3 hr; six rinses in water; immersion in TCH for 30 min; six rinses in water; immersion in 2% osmium tetroxide for 3.5 hr; six rinses in water. In some instances the above procedure was abbreviated by omitting the last four steps.

Method 2. Whole adults were fixed initially in 4% acrolein and 2% glutaraldehyde in 0.1 M sodium cacodylate for 5 hr at room temperature. The specimens were then transferred to 2% glutaraldehyde in the same buffer for storage in a refrigerator for two months. Prior to osmication, the animals were decapitated and the heads rinsed in buffer for at least 1 hr. The remainder of the processing procedure was similar to that described in the preceding paragraph.

Method 3. Whole adult shrimp were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate for 18 hr at room temperature and then refrigerated for five months in the same solution. Prior to osmication, the shrimp were decapitated and the heads rinsed in buffer overnight. The tissue was osmicated by the lengthy procedure described in the first method.

Nauplii were fixed in 2% osmium tetroxide in 0.1 M sodium cacodylate for 5 hr at room temperature. Next, the specimens were rinsed several times in buffer and stored overnight in cold buffer.

After osmication, all SEM specimens were dehydrated in ethanol and critical-point dried with carbon dioxide. Finally, the dried tissue was mounted on aluminum stubs with tape or silver conductive paint and coated with gold in a sputtering device (Technics, Inc.). Specimens were examined and photographed in a Hitachi HHS-2R scanning electron microscope.

Transmission electron microscopy (TEM)

Observations undertaken with TEM were limited in scope and served mainly to clarify the organization of the cuticular layer found at the base of the adult sensilla. Heads of adult brine shrimp were fixed for 15 to 16 hr in either of the following two solutions: 2% acrolein, 5% glutaraldehyde in 0.12 M sodium cacodylate, or 2% acrolein, 4% glutaraldehyde in 0.12 M sodium cacodylate. The tissue was then rinsed for 4.5 hr in 0.2 M sucrose in 0.12 M sodium cacodylate, post-fixed for 2 hr in 2% osmium tetroxide in 0.1 M sodium cacodylate, dehydrated in ethanol, and embedded in Epon epoxy resin. Ultra-thin sections were cut with a diamond knife, stained sequentially with aqueous uranyl acetate and lead citrate, and examined in an RCA EMU 2A or 2C electron microscope.

Dye-penetration experiments

The permeability of antennular sensilla was investigated by means of Slifer's (1960) crystal violet method, a technique that has been used in identifying chemo-

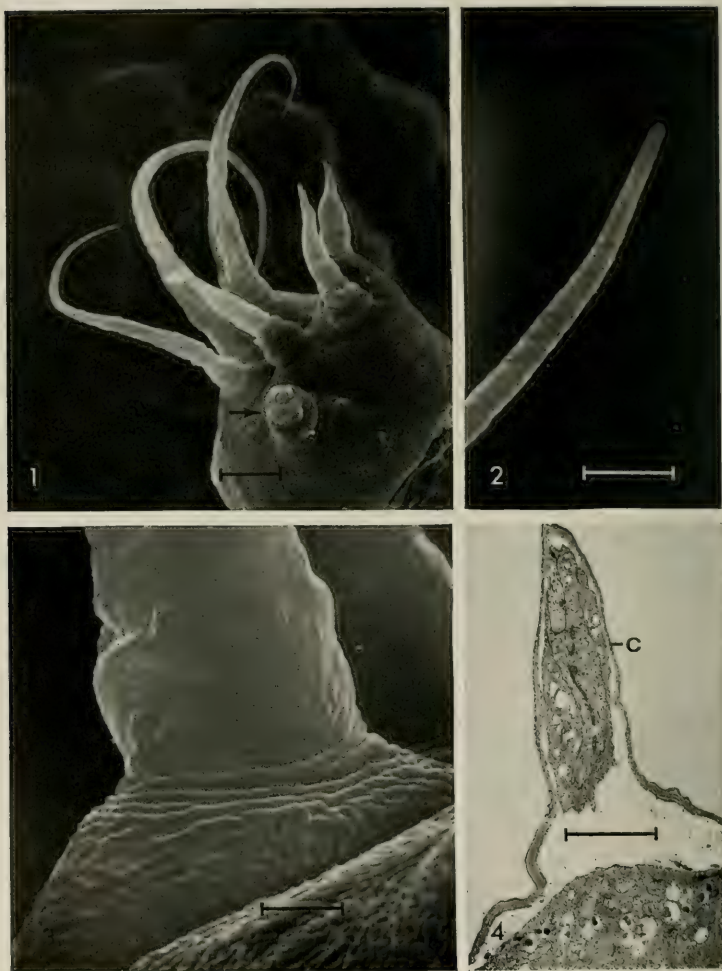


FIGURE 1. Scanning electron micrograph of tip of antennule of adult brine shrimp, showing two types of sensilla. Note three long, curved sensilla (type 1) and three shorter sensilla (type 2). The shaft of a fourth type 2 sensillum is missing, and only the basal knob-like elevation remains (arrow). Bar is 10 μ m.

receptors in insects (Slifer, 1970). Whole adult shrimp were fixed in 3.7% formaldehyde in 0.1 M sodium cacodylate for 30 hr, washed overnight in water, and immersed in 0.5% crystal violet for periods of time varying from 1 min to 14 hr. Each animal was rinsed quickly in two or three changes of water to remove excess dye, and then the antennules were examined immediately by light microscopy. Nauplii were studied in the same way, except that fixation was carried out in a 3.7% formaldehyde solution made with hatch water.

RESULTS

Electron microscopy: adult brine shrimp

The tip of each antennule of an adult *Artemia* possessed a terminal cluster of sensory setae (Fig. 1). Two morphologically distinct kinds of sensilla, here designated type 1 and type 2, were present within a cluster. Type 1 sensilla were longer (43 to 80 μm in length) and, as seen in scanning electron micrographs, simpler in external morphology (Figs. 1-3). They were greatest in diameter at the base, where they originated from the tip of the antennule. Here, where the cuticle of the sensillum became continuous with the cuticle of the antennule proper, there appeared to be no socket or any other kind of articular specialization, although the cuticle of the sensillum and antennule did differ in surface rugosity. Transmission electron micrographs of ultra-thin sections also failed to reveal the presence of any articular modification of the cuticle at the base of the type 1 sensillum (Fig. 4). When the same kind of sensillum was examined more distally by SEM, the shaft of the sensory seta was seen to decrease very gradually in diameter and terminate as a finely tapered tip, which lacked pores or other salient morphological features capable of being resolved by scanning electron microscopy.

Type 2 sensilla (Figs. 1, 5-7) differed from the type 1 sensory setae in a number of respects. Type 2 sensilla were much shorter; the shaft length, as seen in scanning electron micrographs, was only 12 to 23 μm . The base of the type 2 sensillum possessed a distinctive modification of the cuticle in the region of the origin of the shaft. By SEM the shaft appeared to originate from the center of a knob-like bulge of the antennule proper. Transmission electron micrographs of sectioned material revealed that the shaft was in fact recessed within the knob-like bulge (Fig. 7) and that inside the knob the cuticle of the shaft became continuous with a circular invagination of antennular cuticle. Moreover, the cuticle inside the cup-like invagination was much thicker than that of the nearby antennular surface (Fig. 7).

FIGURE 2. High magnification micrograph of distal end of type 1 sensillum of adult. Compare to Fig. 6. Bar is 2 μm .

FIGURE 3. Scanning electron micrograph of basal region of type 1 sensillum of adult. Compare to Fig. 4. Bar is 2 μm .

FIGURE 4. Transmission electron micrograph of oblique section through basal region of type 1 sensillum of adult. Note lack of any articular specialization where cuticle (c) of sensillum becomes continuous with cuticle of antennule proper. Bar is 5 μm .

The shaft of a type 2 sensillum differed from that of a type 1 sensory seta in possessing a shallow, annular furrow (Fig. 5) and a pore on the distal end (Fig. 6). The pore rim (average pore diameter, $0.4\text{ }\mu\text{m}$) was highly variable in morphology from one sensillum to another. The edge of each pore displayed distinctive finger-like or leaf-like cuticular projections that varied in number, length, shape, and arrangement. No two sensilla appeared to be identical in this respect.

Every *Artemia* antennule examined in detail by SEM was found to possess three of the type 1 sensilla; however, the number of type 2 sensilla per terminal cluster was variable. Usually four or five of the latter were present, but one of



FIGURE 5. Scanning electron micrograph of type 2 sensilla of adult. Note the basal knob-like elevation (k) from which arises the shaft (s) of the sensillum. Arrows point to shallow, annular indentation of cuticle of shaft. Bar is $4\text{ }\mu\text{m}$.

FIGURE 6. High magnification micrograph of distal end of type 2 sensillum. Note single pore on tip, and compare to Figure 2. Bar is $0.5\text{ }\mu\text{m}$.

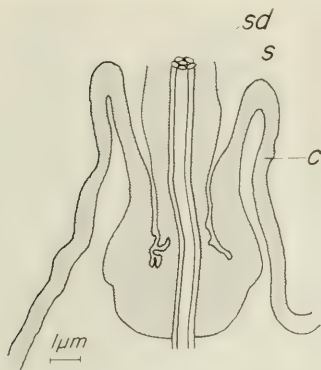


FIGURE 7. Diagram of base of type 2 sensillum as seen in thin section. Note the invagination of cuticle (c) within the basal knob-like elevation, as well as the recessed position of the proximal end of the shaft (s). Sensory dendrites (sd) are situated in lumen of shaft.

the antennules studied had only three. The number of type 2 sensilla per antennular cluster did not appear to be related to the sex of the individual, since totals of four and five were observed on antennules of both males and females.

Both types of sensilla were susceptible to damage or breakage during the protracted processing procedures used to prepare SEM specimens. However, even when a sensillum had broken off near the base of the shaft, it was relatively easy to recognize the site and type of the sensillum by the nature of the stump that remained. The numbers of sensilla reported in the preceding paragraph refer not to the number that remained intact after SEM processing but rather to the number of intact sensilla plus the number of damaged ones. Nothing is known about the percentage of damaged or missing sensilla normally present in the living state, but light microscopic observations of formalin-fixed animals indicated that much of the specimen damage observed by SEM occurred after the primary fixation step.

Electron microscopy: nauplii

The tip of each antennule of an *Artemia* nauplius normally bears three setae (Figs. 8–11). These larval setae varied in length from 13.5 μm to 66 μm and shared a number of features with the type 1 sensilla of adults. These features were: their number and general shape; the apparent absence of an articular modification of the cuticle at the base of the seta; and the absence of a pore at the distal end. Unlike type 1 sensilla of adults, however, the larval setae possessed short spines that were sparsely distributed over the general surface of the seta (Fig. 10). Such spines were sometimes situated quite close to the distal end of the seta.

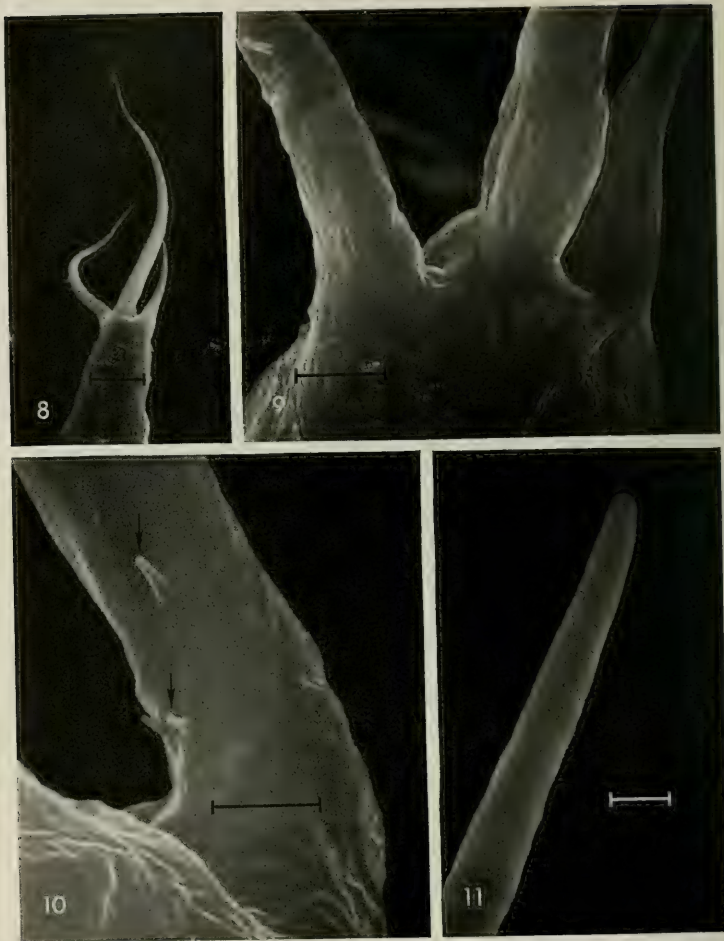


FIGURE 8. Scanning electron micrograph of tip of naupliar antennule (a). Note number and shape of antennular setae. Bar is 10 μ m.

FIGURE 9. Tip of naupliar antennule showing basal region of naupliar setae. Bar is 3 μ m.

FIGURE 10. Basal region of naupliar seta. Note short spines (arrows). Bar is 2 μ m.

FIGURE 11. High magnification micrograph of tip of naupliar seta. Compare to Fig. 2. Bar is 0.5 μ m.

Dye-penetration experiments

Light microscopic examination of antennules of adults immersed in aqueous crystal violet indicated that type 2 sensilla were permeable to the dye, whereas type 1 sensilla were not. At the shortest immersion time studied, *i.e.*, 1 min, the type 2 sensilla were stained, but even after immersion for as long as 14 hr, there was no evidence that crystal violet penetrated the type 1 setae.

With regard to naupliar larvae, there was no indication that the dye was able to enter the cuticle of the larval antennular setae. In some instances staining of the setae was seen, but all the observations made indicated that this staining occurred not by local penetration of the dye but rather by penetration elsewhere on the body surface, followed by internal diffusion of the dye to the antennules. Therefore, the larval setae resembled type 1 sensilla of adults in that both were impermeable to crystal violet solution.

DISCUSSION

During the past decade, scanning electron microscopy has been used to study the external morphology of cuticular sensilla of members of the following major groups of crustaceans: decapods (Ball and Cowan, 1977; Mauchline, Aizawa, Ishimaru, Nishida, and Marumo, 1977; Shelton and Laverack, 1970; Snow, 1974; Tazaki, 1977; Tazaki and Ohnishi, 1974; Thomas, 1971; Vedel and Clarac, 1976), amphipods (Dahl, 1973a, b; Dahl, Emanuelsson, and von Mecklenburg, 1970), isopods (Nielsen and Strömberg, 1973), mysids (Guse, 1978), barnacles (Foster and Nott, 1969; Mumm, Klepal, and Barnes, 1974), copepods (Fleminger, 1973; Strickler, 1975; Strickler and Bal, 1973), ostracods (Danielopol, 1971), and cladocerans (Dahm, 1976). These SEM studies have increased our knowledge of the morphological diversity that exists among crustacean sensilla, but for most of the sensilla examined there are no conclusive physiological data concerning function. The same is true for the antennular setae of brine shrimp. The following discussion of the possible functions of these structures is speculative, and it should be remembered that the specific functions of the antennular sensilla of *Artemia* are not known and at the present time can not be deduced with any certainty from morphological features alone.

In the present study, a sensory function has been assumed for type 1 and type 2 antennular setae of adults because both kinds are known to be innervated (Tyson, unpublished observations). Evidence that the antennules of adult brine shrimp are mechanosensitive comes from a study by Lent (1977), which deals with the coordination of metachronal limb movements in male-female pairs. Lent found that pulsatile water movements directed to the head of a brine shrimp could elicit a change in the metachronal frequency of limb movements of that individual and that pulse responsiveness was lost when both antennules were removed. It is feasible that either type 1 or type 2 sensilla are the mechanoreceptors that Lent suggested are involved in coordinating movements during tandem swimming. In mechanoreception the primary step in the transduction process is generally thought to involve mechanical deformation of a sensory cell process (Thurm, 1965). Deflection or bending of either type 1 or type 2 sensilla could conceivably result in the appropriate stimulation of sensory dendrites. In the case of type 2

sensilla, the presence of a basal articular modification may be significant, since it is possible that this structural specialization provides some flexibility to the base of the shaft. Movement at the site of articulation might result in deformation of the sensory dendrites that enter the shaft in this region. It is also plausible, however, that some flexibility exists at the base of the sensillum without producing concomitant mechanical stimulation of sensory processes. In this regard, it should be pointed out that socket-like modifications have been observed at the base of certain crustacean aesthetascs, and these sensilla are thought to be chemosensory in function (Snow, 1973, 1974).

The type 2 sensilla of adult *Artemia* may in fact be chemoreceptors. Both the presence of a terminal pore and the demonstrated permeability to crystal violet support such a hypothesis. In the case of insect cuticular sensilla, the presence of these two features is considered to be strongly diagnostic of a chemosensory function (Slifer, 1970). In the case of crustaceans, however, it is not clear how indicative of chemoreception the presence of a pore is, because pores have been observed in setae suspected to be mechanosensory, rather than chemosensory (Snow, 1974). In the preceding discussion the possibility has been explored that type 2 sensilla may be either mechanoreceptors or chemoreceptors. It is also feasible that type 2 sensilla are dual in function and each serves in both mechanoreception and chemoreception. In crustaceans the presence of sensilla with a dual function has not been documented, but such sensilla are known to occur in insects (McIver, 1975).

With respect to the nauplii of *Artemia*, it has long been recognized that naupliar antennules bear setae (Anderson, 1967; Joly, 1840), but it is not known whether or not these larval structures are innervated. Nor is anything known about how they are related, in a developmental sense, to the antennular setae of adults. In the present study, naupliar antennules were examined by SEM in order to permit a comparison of the larval and adult structures. The naupliar setae bore no resemblance to the type 2 sensilla of adults, but they were similar to type 1 sensilla in overall shape, in lacking a terminal pore, and in being impermeable to crystal violet. Another salient feature common to both the naupliar setae and the adult type 1 sensilla was their number per antennule. A total of three setae was normally present on each naupliar antennule, and every adult antennule examined by SEM likewise possessed three type 1 sensilla. The type 2 sensilla of adults were, on the other hand, more variable in number, and typically there were four or five present per antennule. It is possible that the three naupliar setae represent a developmental stage in the formation of three of the adult sensilla. If this be true, all available observations support the hypothesis that the larval structures are developmentally related to type 1 sensilla, rather than to type 2.

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SUMMARY

1. Scanning electron microscopy was used to characterize the external morphology of setae found on the antennules of adults and nauplii of the brine shrimp, *Artemia salina* (L.). The permeability of the antennular setae was studied by means of Slifer's crystal violet method.

2. Each antennule of an adult brine shrimp possessed a terminal cluster of sensory setae. Within a cluster there were two morphologically distinct kinds of sensilla, here designated type 1 and type 2. Three type 1 sensilla were observed on every antennule examined. The number of type 2 sensilla per antennule was usually four or five.

3. Type 1 sensilla of adults were 43 to 80 μm long and simple in external morphology. They were widest at the base, decreased in diameter gradually, and terminated at a finely tapered tip. No pores were resolved by scanning electron microscopy.

4. Type 2 sensilla of adults were shorter (shaft length, 12 to 23 μm) and displayed a single pore at the tip (average pore diameter, 0.4 μm). In thin section they were seen to possess a distinctive articular specialization of the cuticle at the base of the seta.

5. Dye penetration experiments indicated that type 2 sensilla were permeable to aqueous crystal violet, whereas type 1 sensilla were not.

6. The antennular setae of nauplii resembled type 1 sensilla in general shape, in being impermeable to crystal violet, and in lacking a terminal pore and basal articular specialization. Moreover, a total of three setae was normally present on each naupliar antennule, and the same number of type 1 sensilla was found on each adult antennule examined. If the three naupliar setae represent a developmental stage in the formation of three adult sensilla, available observations suggest that the larval setae are developmentally related to type 1, rather than to type 2 adult sensilla.

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DEVELOPMENT OF VESTIGIAL TAIL MUSCLE ACETYLCHOLINESTERASE IN EMBRYOS OF AN ANURAL ASCIDIAN SPECIES¹

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Ascidians (subphylum Urochordata, class Ascidiacea) are sessile, filter feeding animals which ordinarily develop a swimming larva for dispersal and site selection. Among the exceptions are a few species of the family Molgulidae that develop an anural larval form during embryogenesis (Lacaze-Duthiers, 1874; Damas, 1902; Berrill, 1931). These species live on sand and mud flats where they encounter no problem of site selection and have a limited need for dispersal. Accordingly, development of such typical tadpole features as adhesive organs, brain sensory structures, tail, notochord, and tail muscles has been suppressed. The embryo hatches into a tailless larva which drifts briefly and comes to rest on the flat. Phylogenetic relationships establish clearly that anural larvae are a secondarily acquired specialization (Berrill, 1931, 1955).

Ascidian development is of unusual interest because a determinate cleavage mechanism produces a "mosaic" of invariable cell lineages for different larval characteristics; surgically isolated partial embryos have a developmental fate determined largely, but not exclusively, by the lineages of the isolated cells (Reverberi, 1961, 1971). This considerable autonomy of expression in normal development indicates that some larval features are independently regulated in development, and conceivably arose independently in evolution. An example reported here, of vestigial acetylcholinesterase (AChE) expression in the presumptive tail muscle tissues of an anural species, offers some further insight into the general character of larval development.

A localized AChE develops in the larval tail muscle cells of urodele ascidian species (Durante, 1956, 1959), but not in the nervous system of most species until after postmetamorphic differentiation of the zooid. Muscle AChE first develops at the neurula stage and persists until the eventual destruction of the tail muscle cells occurs after metamorphosis (Durante, 1956). Surgically isolated muscle lineage blastomeres do not give rise to cells with organized muscle fibrils (Pucciminafra and Ortolani, 1968), but there is a very strong autonomous development of their AChE (Durante, 1957; Whittaker, Ortolani and Farinella-Ferruzza, 1977). Since no obvious superficial features of larval muscle differentiation occur during development in three anural molgulid species, *Molgula arenata*, *Molgula occulta*, and *Bostrichobranchus pilularis* (Berrill, 1931), their embryos and larvae were

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examined histochemically for vestiges of AChE. One of these, *M. arenata*, undergoes a modest development of AChE in the tail muscle rudiment.

MATERIALS AND METHODS

Most of the work described here was done with *Molgula arenata* Stimpson. This and *Bostrichobranchus pilularis* (Verrill) were dredged from sand flats near Senator Shoal in northern Nantucket Sound at Cape Cod, June through November. *Molgula occulta* Kupffer was dredged from sand flats in the vicinity of Roscoff village on the coast of Brittany (France), July and August. Certain control observations were made on species with urodele larvae: *Molgula manhattensis* (DeKay) obtained in the vicinity of Falmouth, Massachusetts, *Molgula occidentalis* Traustedt of the Florida Gulf purchased from the Gulf Specimen Company, Panama, Florida and *Molgula impura* Heller collected at Porticello harbor in northwestern Sicily on the Gulf of Palermo.

Embryo culture

Embryos were cultured in filtered sea water at $18 \pm 0.1^\circ \text{C}$ using a refrigerated constant temperature water bath (Wilkens-Anderson Lo-Temp). In all cases, eggs and sperm were removed surgically by dissection of the gonads and gonoducts, filtered through surgical gauze, and mixed together in a mass culture from which sperm could be decanted. In some molgulid species (*M. arenata* and *B. pilularis*) there was little or no fertilization of eggs obtained in this way unless the eggs remained in contact with sperm suspension for 40–60 min. Eggs of the other species became polyspermic if they were not removed immediately from the sperm suspension. Filters covered with #21 (185 mesh per lineal inch) swiss silk bolting cloth were used for this purpose.

Eggs of all the molgulid species studied still retained their germinal vesicles at the time they were removed from the animal. Germinal vesicle breakdown occurred some time after egg liberation and fertilization took place after the first meiotic division. Final maturation of the egg after shedding into sea water has been noted for a number of ascidian species, although the egg in some species matures in the oviduct (Hirai, 1968). Fertilization time was variable among groups of *M. arenata* eggs, as reflected in the time of first cleavage occurring 80–120 min after the initiation of the cultures. Variability was found with the other species as well. For this reason, time after the 2-cell stage is used in this paper as the basis for reporting comparative sequences of developmental events.

Measurements of egg size

A Nikon Model 2 filar micrometer eyepiece was calibrated (at $250\times$) against two different stage micrometers and used to measure egg diameters, exclusive of the chorionic membrane. Measurements were made from seawater drops without overlying coverslips. The egg sizes of three species, *M. occidentalis*, *M. arenata*, and *M. manhattensis*, are reported in Table I. Two of these measurements differ significantly from those cited by Berrill (1931, 1937). He has stated that all three species have an egg diameter of $110 \mu\text{m}$.

Histology

Embryos and larvae were fixed for 90 min in Bouin's fixative. They were embedded in Paraplast tissue embedding medium (melting point 56–57° C), sectioned at 7–8 μ m, and stained with eosin and Mayer's hematoxylin.

Histochemistry

AChE was localized histochemically by the direct coloring reaction of Karnovsky and Roots (1964) after fixation of embryos and larvae for 3 min in cold (5° C) 80% ethanol (Durante, 1956). Incubation was at 18° C for up to 12 hr. Specimens were handled whole, and afterwards dehydrated, and mounted in damar resin. Histochemical fixation and processing causes a 30–40% shrinkage in embryo diameter between the mounted specimens and live embryos. This accounts for the variable size differences evident in the photographic illustrations.

Acetylthiocholine iodide at 1.7 mM is the reaction substrate. Three other cholinesterase substrates were also used at this concentration: acetyl- β -methylthiocholine iodide, butyrylthiocholine iodide and propionylthiocholine iodide. In addition, the enzyme reaction was tested for its response to the pseudocholinesterase inhibitor tetraisopropylpyrophosphoramidate (iso-OMPA) and Burroughs-Wellcome AChE inhibitor BW 284 C51 dibromide. The substrates and inhibitors were obtained from Sigma Chemical Company and ICN Pharmaceuticals, Inc.

Tyrosinase (dopa oxidase) was localized histochemically in embryos and larvae by the method described by Whittaker (1973a).

Quantitative histochemistry

One can estimate quantitatively the AChE activity in equivalent histochemical preparations by colorimetric assay of the relative rates of reaction product accumulation. Concentrations of the insoluble reaction product of the Karnovsky and Roots (1964) procedure, cupric ferrocyanide, obey the Beer-Lambert law. The quantity of this product can therefore be measured in histochemical specimens by techniques of microdensitometry (Storm-Mathison, 1970; Wenk, Krug and Fletcher, 1973).

Measurements were made on damar mounted ascidian larvae with a Vickers M85 integrating scanning microdensitometer at 485 nm by the subtractive method. Partially developed embryos on the same slide which had not developed AChE were used as blanks. Machine optical density readings were converted to absolute integrated optical density units by calibrating the instrument against a No. 96 Kodak 1.00 Neutral Density filter (10.0% transmission).

RESULTS

Amural development of M. arenata

The general morphological structure of the *Molgula arenata* egg with its closely adhering chorion and barely noticeable small and flattened outer follicle cells, has been described previously by Berrill (1931). As seen in the present study the egg was moderately yolky and opaque, but perhaps only slightly more so than eggs of the other molgulid species examined during this investigation.

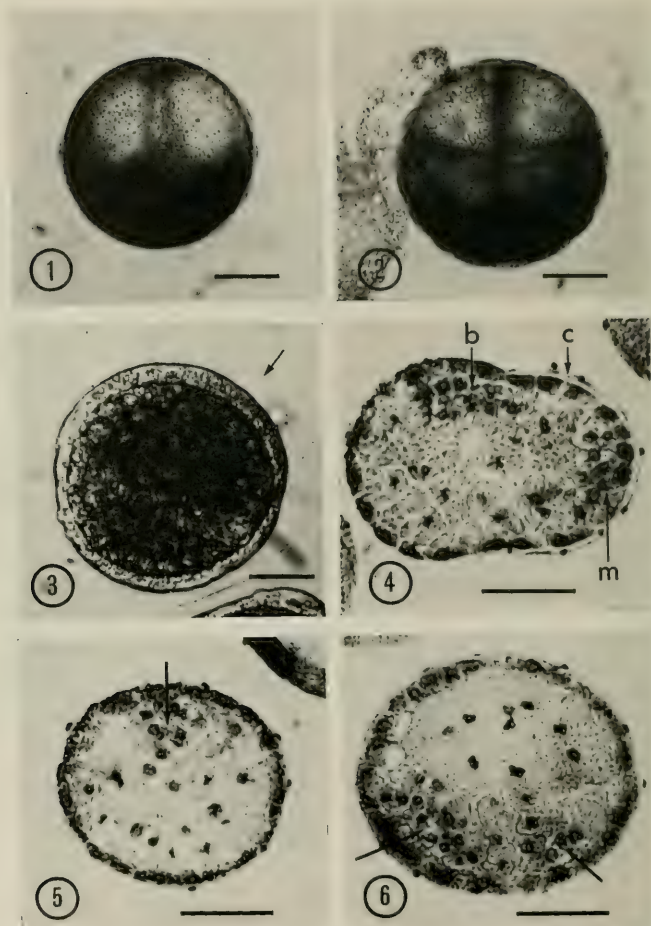


FIGURE 1. Live *Molgula arcuata* egg at the beginning of the first cleavage furrow. Dense yolk granules occur at the vegetal pole. The bar in each figure is 40 μm.

FIGURE 2. Lateral view of the 8-cell stage along the animal-vegetal axis showing two cells of the more yolk-free animal quartet.

FIGURE 3. Hatched live *M. arcuata* larva with yolk-free ectodermal covering and transparent larval test (arrow).

Egg color was light reddish brown with a range that varied through shades of brown to dark gray. A few minutes before the first cleavage furrow became visible there was a sweeping of yolk granules to the lower vegetal hemisphere of the egg, leaving a clear cytoplasmic region at the animal pole (Fig. 1). After third cleavage (8-cell) the animal quartet of cells, which are destined to become the future ectodermal and neural tissues of the larva, was relatively free of yolk (Fig. 2). Cleavage followed the same pattern as in other ascidian embryos (Conklin, 1905; Berrill, 1931). However, the chorion is applied so tightly to the egg that a mild distortion caused by it rendered observation of gastrulation and neurulation features in the living embryo impossible.

About 11.5 hours after first cleavage (at 18° C) slightly oblong larvae without tails or other superficial tadpole features began hatching from the eggs. Surrounding the newly-hatched larva was a smooth transparent test or tunic (Fig. 3) similar to that found around urodele ascidian larvae. Hatching occurred by what appeared to be lysis of one small part of the chorionic membrane: the pressure of the developing test apparently caused the ruptured chorion to retract over the surface of the larva, giving an impression that the larva actively squeezed through a hole in the chorion. A clear and relatively yolk-free ectodermal layer of tissue surrounded an internal endodermal mass of yolky tissue. The larva was essentially characterless and featureless. Although presumptive muscle tissues exist, neither they nor any remnant of the tail cells were visible in the living larva.

Histological sections of larvae revealed two important internal structures: a discrete brain in the anterior or "head" region and a crescent of two nearly contiguous bands of myoblasts in the posterior "tail" part of the larva. Neither of these structures was very highly differentiated. No myofibrils were observed in the histological sections of presumptive muscle. An interesting feature of hatching in *M. arenata* was that the head region of the larva always emerged from the chorion first; this will be more evident in studies of AChE development. Since ascidian embryos have a proteolytic hatching enzyme (Berrill, 1929; Denucé, 1975) the enzyme is probably produced by the head ectodermal cells of the *M. arenata* larva. A sagittal section along the anterior-posterior axis of a hatching larva indicates the positions of the brain and part of the mesodermal crescent (Fig. 4). Cross sections clearly show a brain in the head region (Fig. 5), and the two arms of the mesodermal crescent in the larval posterior (Fig. 6). Similar structures were observed by Damas (1902) in histological sections of larvae of another anural species, *Molgula bleizi* Lacaze-Duthiers.

One finds in the sectioned larvae a single-celled layer of smaller ectodermal cells surrounding an internal mass of quite large endodermal cells. Cytological differences in yolk content are evident in the photographs (Figs. 4-6); the endodermal tissues are of greater density and quite eosinophilic. Both the neural and mesodermal tissues were noticeably less yolky than the endodermal cells, and very

FIGURE 4. Sagittal histological section (8 μ m) along the anterior posterior axis of hatching *M. arenata* larva. Brain (b), mesodermal crescent (m), and egg chorion (c) are evident.

FIGURE 5. Cross section through an *M. arenata* larva in the brain region (arrow).

FIGURE 6. Cross section through an *M. arenata* larva in the region of the mesodermal crescent (arrows).

obviously more basophilic. The presumptive notochordal cells in the region of the mesodermal crescent were neither necrotic nor otherwise distinguishable from the yolk laden endodermal cells of that region. Damas (1902) observed that notochord tissue in *M. bleizi* formed a placode distinct and separate from the endoderm.

The tail phagocytes depicted in drawings of anural larvae by Lacaze-Duthiers (1874) and Berrill (1931) have not been observed in the present study. They were presumed by these authors to be necrotic remnants of the aborted tail cells. Various kinds of optical microscopy (bright field, dark field, phase contrast, and Nomarski differential interference contrast) applied to living material have failed to reveal these cells either in newly-hatched larvae or in postmetamorphic larvae already undergoing formation of the zooid. Cells corresponding to these tail phagocytes have not been observed in histologically sectioned larvae.

The urodele features of development do not cease at some distinct morphological stage in the anural embryo. The two presumptive muscle bands occur in a relationship that is found in the neural plate stage of urodele species, but there is no histological evidence of a notochordal tissue differentiation which would ordinarily become quite evident by this stage. Neural development of the anural larva is characteristic of what one would call an early tailbud stage of embryogenesis, namely, a stage after neurulation at which there is already tail morphogenesis involving the organization of muscle and notochordal tissues. The three larval tissues (brain, tail muscle, notochord) have therefore not all stopped their development at a single morphological stage or development time.

Comparisons with urodele larval development

One sees readily from the sectioned *M. arcnata* larvae that differentiation of the tail tissues failed at a very early stage in their formation. The modest development of the brain is a partial exception to this. Since the adult cerebral ganglion arises from part of the urodele larval brain (Willey, 1893), formation of a complete brain primordium in the anural larva may be essential for later development of the zooid ganglion.

The *M. arcnata* brain did not, however, develop the most striking molgulid larval feature, the otolith melanocyte; there was no trace of otolith pigmentation. This cell, part of a gravity receptor, begins to differentiate melanin pigment during a middle tailbud stage of urodele development. Nor was there any trace of tyrosinase (dopa oxidase), the enzyme responsible for producing ascidian melanin pigment (Minganti, 1951; Whittaker, 1973a), when it was looked for histochemically in whole embryos at various times between 5 and 10 hr of development. The tyrosinase of urodele embryos, *M. occidentalis* and *M. manhattensis*, began to be synthesized at a relatively early stage, several hours before the beginning of melanin pigmentation (Fig. 7).

Larval hatching times of the two urodele molgulids, *M. occidentalis* and *M. manhattensis*, were very close together in spite of a striking difference in egg volume (Table I); there was only 30 min difference in their hatching time (Fig. 7). The *M. arcnata* egg, which falls within the size range of the two urodele species, actually began hatching earlier by 2–2.5 hr than either urodele species. This modest abbreviation of development time undoubtedly follows from the curtailment of larval

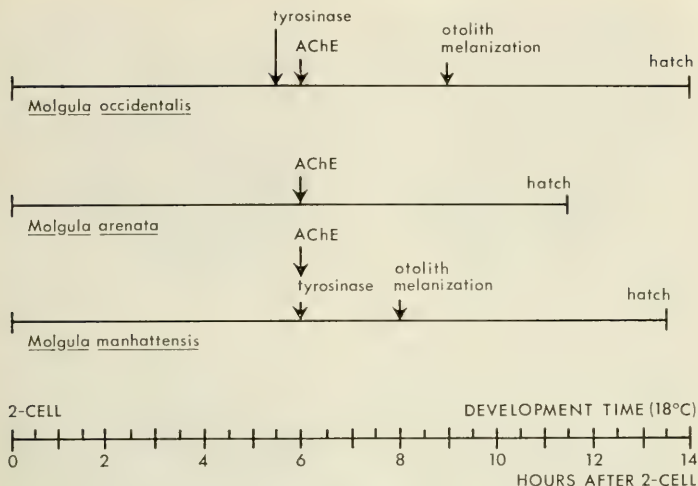


FIGURE 7. Time of first acetylcholinesterase (AChE) synthesis and other events in three molgulid species.

expression and reflects the relative non-importance of zooid development during molgulid embryogenesis. Molgulid larvae are notable in lacking morphological traces of zooid structure at the time of hatching.

There was a great similarity in the details and timing of postmetamorphic development in *M. arenata* and the urodele species. Morphological comparisons have been illustrated previously by Berrill (1931) for *M. arenata* and *M. manhattensis*. In the present study, the time taken under laboratory conditions for these species to produce a functional zooid was long, about 5 days; there was no significant difference between *M. arenata* and the two urodele species.

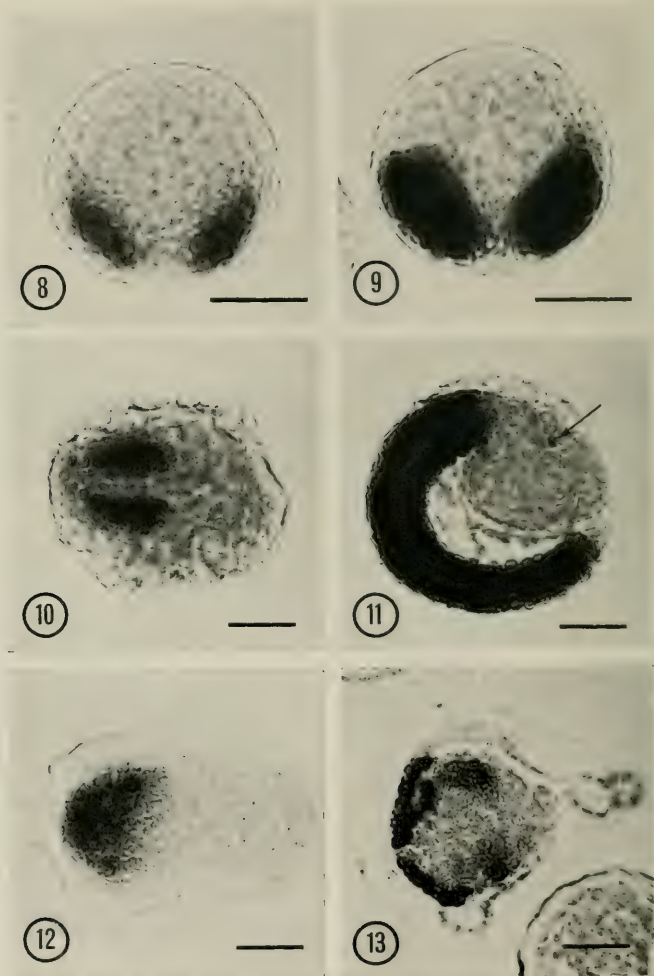
Acetylcholinesterase in M. arenata

M. arenata embryos began to make a histochemically detectable AChE at approximately 6 hr after the 2-cell stage. This was also the same time at which

TABLE I

Egg size, exclusive of the chorionic membrane, in three *Molgula* species. Mixed eggs from a group of 3-10 animals; $N = 50$.

Species	Diameter ($\mu\text{m} \pm \text{s.e.}$)	Volume ($\mu\text{m}^3 \times 10^{-3}$)
<i>Molgula occidentalis</i>	141 ± 1	147
<i>Molgula arenata</i>	132 ± 1	121
<i>Molgula manhattensis</i>	114 ± 1	78



FIGURES 8-13. Various developmental stages and species stained histochemically for acetylcholinesterase. The bar in each figure is 40 μ m.

FIGURES 8 AND 9. *M. arenata* larvae.

AChE activity first occurred in the two urodele species (Fig. 7). Enzyme activity in *M. arenata* first appeared as a faint staining which increased gradually in intensity over about 3 hr and then appeared to remain constant up to and after hatching. There was a noticeable range of staining intensity in the hatched larvae: most had a distinct modest staining (Fig. 8), but a few within each group stained relatively strongly (Fig. 9). The number of larvae hatching out in the various cultures correlated roughly with the number of embryos in those cultures having AChE activity. There appeared to be few, if any, larvae that did not make some AChE.

Localization of AChE activity in a peripheral broken crescent of cells is further evidence that the mesoderm cells in *M. arenata* have remained throughout development in a morphological configuration characteristic of neural plate and neurula stages. Comparison with *M. occidentalis* (Figs. 10, 11) and *M. manhattensis* (not shown) illustrates that activity in urodele embryos first occurred at a neurula stage when there were already parallel rows of myoblast cells (Fig. 10). As the tail became better formed there was increasing activity in the muscle tissues (Fig. 11). The chronological age at first enzyme occurrence in *M. arenata* corresponds to what would be a neurula stage in urodele development. Myoblast number in the *M. arenata* larvae also resembles a stage much later than gastrulation. There were at least 38 AChE-containing cells in the larvae; this is the terminal number of muscle cells in oviparous urodele larvae (Berrill, 1935). Muscle cell lineage divisions in urodele embryos are probably complete by the end of neurulation.

When anural larvae were fixed during the process of hatching and reacted histochemically for AChE, such larvae were found, as in Figure 12, to be emerging head first from the chorion, with the region containing the AChE-reactive myoblast crescent escaping last. This confirms the conclusion reached from observations on sagittal histological sections of hatching larvae (Fig. 4).

What actually happens in *M. arenata* to the AChE-containing myoblasts during postmetamorphic differentiation of the zooid is not clear. The cells become neither necrotic nor phagocytized during early stages of zooid development. They retained their enzyme and remained somewhat dispersed throughout part of the zooid (Fig. 13). Some of these cells may possibly contribute to the developing adult muscle structures; many of them were still present as these muscles formed. If so, this would be most unusual. The muscle cells of the resorbed tails in urodele ascidians usually undergo dissolution and resorption (Cloney, 1978).

Acetylcholinesterase properties

AChE in *M. arenata* larvae had the same responses to certain inhibitors and substrates shown previously for the AChE of *Ciona intestinalis* (L.) (Whittaker *et al.*, 1977) and by these criteria would be considered an "acetylcholinesterase"

FIGURE 10. Late neurula stage *M. occidentalis* embryo.

FIGURE 11. Middle tailbud stage *M. occidentalis* embryo at the time of first otolith pigmentation (arrow).

FIGURE 12. *M. arenata* larva fixed at time of hatching.

FIGURE 13. *M. arenata* larva at the beginning of zooid formation 24 hr after hatching.

(Silver, 1974). The enzyme was inhibited by the AChE inhibitor BW 284 C51 dibromide (1.7 mM) and not by the pseudocholinesterase inhibitor iso-OMPA (3.5 mM). It hydrolyzed acetylthiocholine but not butyrylthiocholine. The muscle cell enzymes of *M. occidentalis* and *M. manhattensis* had similar properties.

Some acetylcholinesterases also hydrolyze propionylcholine (Silver, 1974) and *M. arenata* enzyme proved to be one of these. Propionylthiocholine iodide in the histochemical reaction medium (1.7 mM) gave a strong reaction but not as intense as either acetylthiocholine iodide or acetyl- β -methylthiocholine iodide. *M. occidentalis* AChE essentially did not hydrolyze propionylthiocholine; almost no reaction product was formed. *M. manhattensis* enzyme, however, hydrolyzed propionylthiocholine practically as well as *M. arenata* AChE.

Substrate preferences may have important implications of phylogenetic relationship. AChE in larvae of a southern European species, *Molgula impura*, also had virtually no reactivity towards propionylthiocholine. *M. impura* and *M. occidentalis* have a number of minor character differences (e.g., adult size, body color, egg size) but they are regarded by Monniot (1969) as the same species.

Measurements of acetylcholinesterase

Comparisons of larval enzyme activity were made between *M. occidentalis*, *M. manhattensis* and *M. arenata* by scanning integrating microdensitometry (Table II). Activity measurements were done on whole larvae with partially developed embryos in the same preparations being used as subtraction blanks for non-specific and non-localized background staining. Product was measured in the urodele larvae after 1–3 hr incubation (18° C) and after 10–12 hr incubation for *M. arenata* larvae; activity is expressed as an hourly rate figure. The product accumulation in this material has been found to be linear over several hours, and for at least 12 hr in the lower activity range. For convenience in the use of subtraction blanks (geometrical similarity), the coiled unhatched urodele larvae were fixed for assay 30 min before hatching; measurements were done on *M. arenata* larvae fixed at first hatch. Enzyme activity in the two urodele species was essentially the same (Table II) when the activity values were normalized to the same egg volume using

TABLE II

Acetylcholinesterase activity in larvae of three Molgula species. Activity is expressed as absolute integrated optical density units; mean of 25 randomly selected embryos.

Species	Series No.	Units embryo ⁻¹ hour ⁻¹ $\pm$ s.e.	Normalized* units embryo ⁻¹ hour ⁻¹ $\pm$ s.e.	% of urodele activity**
<i>Molgula occidentalis</i>	1	2,350 $\pm$ 70	1,920 $\pm$ 60	—
	2	2,650 $\pm$ 60	2,170 $\pm$ 50	—
<i>Molgula manhattensis</i>	3	1,140 $\pm$ 60	1,760 $\pm$ 90	—
	4	1,680 $\pm$ 80	2,560 $\pm$ 120	—
<i>Molgula arenata</i>	5	126 $\pm$ 14	—	6
	6	108 $\pm$ 9	—	5

* Normalized to volume of *M. arenata* embryos.

** Based on the mean of series 1–4.

TABLE III

Acetylcholinesterase activity in larvae of Molgula arenata.

	Units embryo ⁻¹ hour ⁻¹ $\pm$ s.e.	% of urodele activity*
Randomly selected group (N = 25)	126 $\pm$ 14	6
Strongest staining larvae (N = 25)	297 $\pm$ 13	14
Range within the combined groups	14-414	0.01-20

* Based on a mean of the urodele AChE activity values (series 1-4) in Table II.

the data of Table I. AChE activity in urodele molgulid larvae appears, therefore, to be proportional to egg (*i.e.* larval) volume. *M. arenata* larvae contained approximately 5-6% of the enzyme activity that would be found in urodele larvae of comparable volume.

There was a range of AChE activity in *M. arenata* larvae, which on a typical slide preparation varied from 0.01-20% of the urodele larval amount (Table III). A randomly selected group of 25 had a mean of 6%. Selection of 25 of the most strongly staining larvae on the same slide, similar to the larva depicted in Figure 9, had a mean of 14% of the urodele activity.

Acetylcholinesterase in other anural species

Larvae of two other anural molgulid species, *M. occulta* and *B. pilularis*, showed no trace of AChE activity. These species also had no vestige of otolith cell melanin or tyrosinase activity. The general aspects of development in these two species are similar to those of *M. arenata* and have been described by Berrill (1931).

Failure to find AChE activity could have many possible trivial causes. Larvae of some urodele species stain more effectively for AChE if fixatives other than cold 80% ethanol are used. Two such fixatives are 4% glutaraldehyde in sea water and 1.5% glutaraldehyde plus 1.5% fresh formaldehyde in 0.2 M cacodylate buffer (pH 7.4) containing 1.0 mM CaCl₂; each used at 5° C for 15 min. Additions of 0.1% Triton X-100 to the incubation medium sometimes enhances the AChE reaction (Gerebtzoff, 1962), and 10% dimethylsulfoxide assists in the demonstration of some enzymes by aiding substrate penetration (Reiss, 1971). These variations of technique all failed to reveal AChE activity in *B. pilularis* larvae.

When small *B. pilularis* adults were dissected free of their tunics and reacted for AChE by the usual technique employed with *M. arenata*, enzyme activity was revealed in siphon and body muscles and in the cerebral ganglion and nerves. Since this technique so readily detects AChE in adult tissues it seems likely, therefore, that *B. pilularis* larvae actually do lack the enzyme.

DISCUSSION

Molgula arenata is distributed along the Atlantic continental shelf from the Gulf of Maine to Georgia (Plough, 1978). The two common urodele species

that overlap the *M. arenata* range are *M. manhattensis* in the north and *M. occidentalis* in the most southern part. On the basis of these species having six folds on each side of the branchial sac, all three might be regarded as closely related (Monniot, 1969). If one assumes that larval characters are among the most conservative features in the course of ascidian evolution, then substrate specificity of the larval AChE indicates a closer phylogenetic relationship between *M. arenata* and *M. manhattensis*. Fortunately, it is not necessary to decide which of the urodele species most closely resembles the progenitor of *M. arenata*.

M. manhattensis and *M. occidentalis* are themselves sufficiently closely related that they have virtually the same amount of larval AChE activity on a size-adjusted basis (Table II). Either urodele species serves equally well for relating the amount of AChE in *M. arenata* to that in a potential urodele progenitor. The 5-6% mean amount is substantial; this is indeed a partial expression and, strictly speaking, not a mere vestige. Individual embryos in an experimental group can have as much as 20% of the urodele AChE activity (Table III).

Programs for the differentiation of larval structures in urodele embryos are controlled, at least in part, by materials laid down in the egg cytoplasm and segregated differentially during cleavage into particular larval tissues and organs (Whittaker, 1979). Some of these must function as activators of specific genetic programs (Davidson, 1976). Larval AChE and tyrosinase development in ascidians appear to be among the larval features regulated by cytoplasmic morphogenetic determinants (Whittaker, 1973b).

Anural embryos have possibly suppressed tadpole characteristics by altering genes responsible for producing the appropriate determinants during oogenesis. This would be a reasonable method of voiding whole patterns of later phenotypic expression without altering structural genes, such as those for AChE, that also presumably function in zooid development. AChE which is absent in the anural larvae of *B. pilularis* is, nevertheless, abundant in the muscles and nerves of the adult zooid, suggesting thereby that no change has occurred in the structural genes for AChE.

The vestigial AChE of *M. arenata* embryos indicates by its presence that different larval systems become inactivated separately, an observation consistent with the historical finding of relative developmental autonomy for the various ascidian tissue lineages and the idea of separately localized morphogenetic determinants. Incomplete and variable suppression of AChE synthesis also suggests the possibility that changes may occur in the quantity of AChE determinant produced. There is already evidence that determinants in the melanocyte lineage are involved in exercising quantitative control over phenotypic expression, perhaps by virtue of their own quantity (Whittaker, 1976).

The relative autonomy in development of many larval features renders it likely that selection against them in the course of environmental adaptation will occur independently. Vestigial enzyme of *M. arenata* in the absence of other larval features effectively attests to this. The autonomy probably also reflects the independent origins of the various larval structures during the original evolution of the ascidian larval form (Berrill, 1955). Selection pressures involved in anural evolution may very well act upon the same genetic systems initially responsible for the acquisition of the motile and sensory functions of tadpole larvae.

The finding that one anural species retains a vestige of larval differentiation whereas others do not might indicate something of the relative age of these anural species; possibly *M. arenata* has evolved from its progenitor species somewhat later than the other two. A consideration of the adult zooid structure (described below) suggests the opposite, however, namely that *M. arenata* probably evolved from a more primitive and ancient stock than did either *M. occulta* or *B. pilularis*. The adult affinities merely suggest possible age differences in the parental stocks; unfortunately they do not indicate how recently the respective anural conditions might have evolved.

One of the striking evolutionary trends within the class Ascidiacea is the change from relative simplicity of adult branchial structures, as in the primitive and ancient family Cionidae, to the more complex structures in the highly evolved and more recently specialized members of the families Pyuridae and Molgulidae (Van Name, 1945; Monniot, 1969; Plough, 1978). Even within such a homogeneous group as the family Molgulidae, there is a marked progression in the degree of infolding of the branchial sac and relative complexity with which the apertures (stigmata) of the branchial sac are spirally arranged into conical infundibulae. In general, the *Molgula* species with the more simplified branchial structures are probably the more ancient and primitive members of the Molgulidae, although secondarily acquired simplifications of branchial structure apparently do occur.

On this basis one would regard *M. arenata* as a relatively older species: it possesses six folds on each side of the branchial sac and its arrangement of the stigmata is fairly simple. *M. occulta* and *B. pilularis* appear to be more recent species. *M. occulta* has seven branchial folds; the branchial sac of *B. pilularis* is without folds but their place is taken by seven stout internal longitudinal vessels that suggest a likely progenitor with seven branchial folds. The infundibulae of *B. pilularis* are the most specialized of all, being in the form of finger-like projections.

Exclusive of the various abyssal ascidian species, which probably do not produce urodele larvae, there are at least nine other molgulids that have evolved anural larval development (Berrill, 1931; Plough, 1978) and two members of the family Styelidae (Millar, 1954, 1962). Some of these larvae undoubtedly retain vestiges of urodele expression at either the morphological or biochemical levels. Investigation of such other species holds the promise of throwing additional light on evolutionary questions and on the ways in which development of ascidian larval characters are determined.

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SUMMARY

1. The ascidian *Molgula arenata* produces an anural larva lacking a tail and other structural features of typical urodele larvae in the family Molgulidae, yet its embryos developed a histochemically detectable acetylcholinesterase in the

tail muscle rudiment. Development of the myoblasts seemed to fail during the neurula stage.

2. Larval enzyme activity occurred at a mean of 5-6% of the level found in the urodele species *Molgula occidentalis* and *Molgula manhattensis*, as measured by scanning integrating microdensitometry of the histochemical reaction product. Some anural larvae had as much as 20% of the enzyme activity in urodele larvae.

3. This example of vestigial expression in the absence of other urodele larval features further illustrates the autonomy of a histospecific enzyme development thought to be controlled by an egg cytoplasmic determinant. Partial suppression of the determinant might be the cause of this diminished expression.

4. Two other anural molgulid species, *Molgula occulta* and *Bostrichobranchus pilularis*, did not have vestigial larval enzyme and possibly have lost the determinant completely.

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 LIONEL I. REBHUN, University of Virginia
 DOROTHY ROWE, Boston, Massachusetts
 W. NICHOLAS THORNDIKE, Boston, Massachusetts
 EDWARD O. WILSON, Harvard University

CLASS OF 1979

ROBERT D. ALLEN, Dartmouth College
 FRANCIS D. CARLSON, The Johns Hopkins University
 HAYS CLARK, Avon Products, Incorporated
 DENNIS FLANAGAN, Scientific American
 PHILIP GRANT, University of Oregon
 CATHERINE HENLEY, National Institutes of Health
 E. SWIFT LAWRENCE, Pawtucket Institution for Savings
 JOHN W. MOORE, Duke University Medical Center
 MELVIN SPIEGEL, Dartmouth College

STANDING COMMITTEES

EXECUTIVE COMMITTEE OF THE BOARD OF TRUSTEES

PROSSER GIFFORD, <i>ex officio</i>	FRANCIS CARLSON, 1980
PAUL R. GROSS, <i>ex officio</i>	PHILIP GRANT, 1980
JONATHAN O'HERRON, <i>ex officio</i>	BENJAMIN KAMINER, 1979
JOHN W. MOORE, 1981	KEITH R. PORTER, 1979
MELVIN SPIEGEL, 1981	

BUDGET COMMITTEE

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JOHN M. ARNOLD	JEROME SCHIFF
GEORGE H. A. CLOWES, JR.	HOMER P. SMITH, <i>ex officio</i>
PAUL R. GROSS, <i>ex officio</i>	WALTER S. VINCENT
WILLIAM T. GOLDEN	

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FRANCIS HOSKIN, <i>Chairman</i>	AUDREY E. V. HASCHEMEYER
FRANK CHILD	FRANK LONGO
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DANIEL GILBERT	ALLEN SCHUETZ
ROBERT GUNNING, <i>ex officio</i>	EVELYN SPIEGEL

COMPUTER SERVICE COMMITTEE

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ALLAHVERDI FARMANFARMAIAN	

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CAROL EBERHARD	DONALD LEHY
CHARLOTTE FRANK	RAYMOND STEPHENS
JUDITH GRASSLE	

HOUSING, FOOD SERVICE AND DAY CARE COMMITTEE

JUDITH GRASSLE, <i>Chairman</i>	BRIAN M. SALZBERG
DANIEL ALKON	HOMER P. SMITH, <i>ex officio</i>
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SAMUEL I. BEALE	LEON P. WEISS
AIMLEE LADERMAN	JANE ZAKEVICIUS

INSTRUCTION COMMITTEE

BENJAMIN KAMINER, <i>Chairman</i>	R. K. JOSEPHSON
DANIEL ALKON	MORTON MASER, <i>ex officio</i>
ROBERT ALLEN	MERLE MIZELL
CLAY M. ARMSTRONG	JOEL ROSENBAUM
ESTHER GOUDSMIT	SHELDON J. SEGAL
ARTHUR HUMES	GEORGE WOODWELL

INVESTMENT COMMITTEE

W. NICHOLAS THORNDIKE, <i>Chairman</i>	MAURICE LAZARUS
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WILLIAM T. GOLDEN	JONATHAN O'HERRON, <i>ex officio</i>

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BERNARD DAVIS	ARTHUR PARDEE
JOHN DOWLING	SHELDON J. SEGAL
J. W. HASTINGS	MARJORIE R. STETTEN
SHINYA INOUE	STANLEY WATSON

MACY SCHOLARSHIP COMMITTEE

JOEL E. BROWN, <i>Chairman</i>	WILLIAM W. SUTTON
ALLEN COUNTER	JAMES TOWNSEL
LOWELL DAVIS	CHARLES WALKER
MORTON MASER, <i>ex officio</i>	

MARINE RESOURCES COMMITTEE

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CYRUS LEVINTHAL	JOHN VALOIS, <i>ex officio</i>
ROBERT PRENDERGAST	

RADIATION COMMITTEE

WALTER S. VINCENT, <i>Chairman</i>	JOHN HOBBIE
EUGENE BELL	E. F. MACNICHOL, JR.
FRANCIS BOWLES	MORTON MASER, <i>ex officio</i>
RICHARD L. CHAPPELL	HARRIS RIPPS
PAUL J. DEWEER	

RESEARCH SERVICES COMMITTEE

ARTHUR DUBOIS, <i>Chairman</i>	T. NARAHASHI
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FRANCIS P. BOWLES	JEROME SCHIFF
S. S. KOIDE	BRUCE SZAMIER
E. F. MACNICHOL, JR.	JAY WELLS
MORTON MASER, <i>ex officio</i>	SEYMOUR ZIGMAN

RESEARCH SPACE COMMITTEE

TIMOTHY GOLDSMITH, <i>Chairman</i>	MORTON MASER, <i>ex officio</i>
JOHN ARNOLD	BRIAN SALZBERG
PHILIP GRANT	ANN STUART
FREDERICK GRASSLE	BETTY WALL
GEORGE LANGFORD	GEORGE WOODWELL

SAFETY COMMITTEE

ROBERT GUNNING, <i>Chairman</i>	E. F. MACNICHOL, JR.
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FRANCIS P. BOWLES	TOSHIO NARAHASHI
LEWIS LAWDAY	RAYMOND E. STEPHENS
DONALD LEHY	FREDERICK THRASHER

II. CERTIFICATE OF ORGANIZATION

(On File in the Office of the Secretary of the Commonwealth)

No. 3170

We, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, and William T. Sedgwick, Edward G. Gardiner, Susan Mims and Charles Sedgwick Minot being a majority of the Trustees of the Marine Biological Laboratory in compliance with the requirements of the fourth section of chapter one hundred and fifteen of the Public Statutes do hereby certify that the following is a true copy of the agreement of association to constitute said Corporation, with the names of the subscribers thereto:-

We, whose names are hereto subscribed, do, by this agreement, associate ourselves with the intention to constitute a Corporation according to the provisions of the one hundred and fifteenth chapter of the Public Statutes of the Commonwealth of Massachusetts, and the Acts in amendment thereof and in addition thereto.

The name by which the Corporation shall be known is THE MARINE BIOLOGICAL LABORATORY

The purpose for which the Corporation is constituted is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history.

The place within which the Corporation is established or located is the city of Boston within said Commonwealth.

The amount of its capital stock is none.

In Witness Whereof, we have hereunto set our hands, this twenty seventh day of February in the year eighteen hundred and eighty-eight, Alpheus Hyatt, Samuel Mills, William T. Sedgwick, Edward G. Gardiner, Charles Sedgwick Minot, William G. Farlow, William Stanford Stevens, Anna D. Phillips, Susan Mims, B. H. Van Vleck.

That the first meeting of the subscribers to said agreement was held on the thirteenth day of March in the year eighteen hundred and eighty-eight.

In Witness Whereof, we have hereunto signed our names, this thirteenth day of March in the year eighteen hundred and eighty-eight, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, Edward G. Gardiner, William T. Sedgwick, Susan Mims, Charles Sedgwick Minot.

(Approved on March 20, 1888 as follows:

I hereby certify that it appears upon an examination of the within written certificate and the records of the corporation duly submitted to my inspection, that the requirements of sections one, two and three of chapter one hundred and fifteen, and sections eighteen, twenty and twenty-one of chapter one hundred and six, of the Public Statutes, have been complied with and I hereby approve said certificate this twentieth day of March A.D. eighteen hundred and eighty-eight.

CHARLES ENDICOTT
Commissioner of Corporations)

III. ARTICLES OF AMENDMENT

(On file in the office of the Secretary of the Commonwealth)

We, James D. Ebert, President, and David Shepro, Clerk of the Marine Biological Laboratory, located at Woods Hole, Massachusetts 02543, do hereby certify that the following amendment to the Articles of Organization of the Corporation was duly adopted at a meeting held on August 15, 1975, as adjourned to August 29, 1975, by vote of 444 members, being at least two-thirds of its members legally qualified to vote in the meetings of the corporation:

VOTED: That the Certificate of Organization of this corporation be and it hereby is amended by the addition of the following provisions:

"No Officer, Trustee or Corporate Member of the corporation shall be personally liable for the payment or satisfaction of any obligation or liabilities incurred as a result of, or otherwise in connection with, any commitments, agreements, activities or affairs of the corporation.

"Except as otherwise specifically provided by the Bylaws of the corporation, meetings of the Corporate Members of the corporation may be held anywhere in the United States.

"The Trustees of the corporation may make, amend or repeal the Bylaws of the corporation in whole or in part, except with respect to any provisions thereof which shall by law, this Certificate or the Bylaws of the corporation, require action by the Corporate Members."

The foregoing amendment will become effective when these articles of amendment are filed in accordance with Chapter 180, Section 7 of the General Laws unless these articles specify, in accordance with the vote adopting the amendment, a later effective date not more than thirty days after such filing, in which event the amendment will become effective on such later date.

In Witness whereof and Under the Penalties of Perjury, we have hereto signed our names this 2nd day of September, in the year 1975, James D. Ebert, President; David Shepro, Clerk.

(Approved on October 24, 1975, as follows:

I hereby approve the within articles of amendment and, the filing fee in the amount of \$10 having been paid, said articles are deemed to have been filed with me this 24th day of October, 1975.

PAUL GUZZI

Secretary of the Commonwealth)

IV. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised August 11, 1978)

I. (A) The name of the Corporation shall be The Marine Biological Laboratory. The Corporation's purpose shall be to establish and maintain a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history.

(B) Marine Biological Laboratory admits students without regard to race, color, sex, national and ethnic origin to all the rights, privileges, programs and activities generally accorded or made available to students in its courses. It does not discriminate on the basis of race, color, sex, national and ethnic origin in employment, administration of its educational policies, admissions policies, scholarship and other programs.

II. (A) The members of the Corporation ("Members") shall consist of persons elected by the Board of Trustees, upon such terms and conditions and in accordance with such procedures, not inconsistent with law or these Bylaws, as may be determined by said Board of Trustees. Except as provided below, any Member may vote at any meeting, either in person or by proxy executed no more than six months prior to the date of such meeting. Members shall serve until their death or resignation unless earlier removed, with or without cause, by the affirmative vote of two-thirds of the Trustees then in office. Any Member who has attained the age of seventy years or has retired from his home institution shall automatically be designated a Life Member provided he signifies his wish to retain his membership. Life Members shall not have the right to vote and shall not be assessed for dues.

(B) The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

III. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk, elected or appointed by the Trustees as set forth in Article IX.

IV. The Annual Meeting of the Members shall be held on the Friday following the Second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 a.m. Subject to the provisions of Article VIII(2), at such meeting the Members shall choose by ballot six Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the Members may be called by the Chairman or Trustees to be held at such time and place as may be designated.

V. Twenty five Members shall constitute a quorum at any meeting. Except as otherwise required by law or these Bylaws, the affirmative vote of a majority of the Members voting in person or by proxy at a meeting attended by a quorum (present in person or by proxy) shall constitute action on behalf of the Members.

VI. (A) Inasmuch as the time and place of the Annual Meeting of Members are fixed by these Bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of Members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least 15 days before such meeting, to each Member at his or her address as shown on the records of the Corporation.

(B) Any meeting of the Members may be adjourned to any other time and place by the vote of a majority of those Members present or represented at the meeting, whether or not such Members constitute a quorum. It shall not be necessary to notify any Member of any adjournment.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated. Notice of Trustees' meetings may be given orally, by telephone, telegraph or in writing; and notice given in time to enable the Trustees to attend, or in any case notice sent by mail or telegraph to a Trustee's usual or last known place of residence, at least one week before the meeting shall be sufficient. Notice of a meeting need not be given to any Trustee if a written waiver of notice, executed by him before or after the meeting is filed with the records of the meeting, or if he shall attend the meeting without protesting prior thereto or at its commencement the lack of notice to him.

VIII. (A) There shall be four groups of Trustees:

(1) Trustees (the "Corporate Trustees") elected by the Members according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Trustees shall be divided into four classes of six, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms.

(2) Trustees ("Board Trustees") elected by the Trustees then in office according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Board Trustees shall be divided into four classes of three, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms. It is contemplated that, unless otherwise determined by the Trustees for good reason, Board Trustees shall be individuals who have not been considered for election as Corporate Trustees.

(3) Trustees ex officio, who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(4) Trustees emeriti who shall include any Member who has attained the age of seventy years (or the age of sixty five and has retired from his home institution) and who has served a full elected term as a regular Trustee, provided he signifies his wish to serve the Laboratory in that capacity. Any Trustee who qualifies for emeritus status shall continue to serve as a regular Trustee until the next Annual Meeting whereupon his office as regular Trustee shall become vacant and be filled by election by the Members or by the Board, as the case may be. The Trustees ex officio and emeriti shall have all the rights of the Trustees, except that Trustees emeriti shall not have the right to vote.

(B) The aggregate number of Corporate Trustees and Board Trustees elected in any year (excluding Trustees elected to fill vacancies which do not result from expiration of a term) shall not exceed nine. The number of Board Trustees so elected shall not exceed three and unless otherwise determined by vote of the Trustees, the number of Corporate Trustees so elected shall not exceed six.

(C) The Trustees and Officers shall hold their respective offices until their successors are chosen in their stead.

(D) Any Trustee may be removed from office at any time with or without cause, by vote of a majority of the Members entitled to vote in the election of Trustees; or for cause, by vote of two-thirds of the Trustees then in office. A Trustee may be removed for cause only if notice of such action shall have been given to all of the Trustees or Members entitled to vote, as the case may be, prior to the meeting at which such action is to be taken and if the Trustee so to be removed shall have been given reasonable notice and opportunity to be heard before the body proposing to remove him.

(E) Any vacancy in the number of Corporate Trustees, however arising, may be filled by the Trustees then in office unless and until filled by the Members at the next Annual Meeting. Any vacancy in the number of Board Trustees may be filled by the Trustees.

(F) A Corporate Trustee or a Board Trustee who has served an initial term of at least 2 years duration shall be eligible for re-election to a second term, but shall be ineligible for re-election to any subsequent term until two years have elapsed after he last served as a Trustee.

IX. (A) The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall elect a Treasurer and Clerk to serve one year, and Board Trustees as described in Article VIII (B). They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers

and agents of the Corporation and may remove them at any time. They may fill vacancies occurring in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number as provided in Article X, and to delegate to such Committee such of their own powers as they may deem expedient in addition to those powers conferred by Article X. They shall from time to time elect Members to the Corporation upon such terms and conditions as they shall have determined, not inconsistent with law or these Bylaws.

(B) The Board of Trustees shall also have the power, by vote of a majority of the Trustees then in Office, to elect an Investment Committee and any other committee and, by like vote, to delegate thereto some or all of their powers except those which by law, the Articles of Organization or these Bylaws they are prohibited from delegating. The members of any such committee shall have such tenure and duties as the Trustees shall determine; provided that the Investment Committee, which shall oversee the management of the Corporation's endowment funds and marketable securities, shall include the Chairman of the Board of Trustees, the Treasurer of the Corporation, and the Chairman of the Corporation's Budget Committee, as *ex officio* members, together with such Trustees as may be required for not less than two-thirds of the Investment Committee to consist of Trustees. Except as otherwise provided by these Bylaws or determined by the Trustees, any such committee may make rules for the conduct of its business; but, unless otherwise provided by the Trustees or in such rules, its business shall be conducted as nearly as possible in the same manner as is provided by these Bylaws for the Trustees.

X. (A) The Executive Committee is hereby designated to consist of not more than ten members, including the *ex officio* Members (Chairman of the Board of Trustees, President, Director and Treasurer); and six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term.

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice Chairman. A majority of the members of the Executive Committee shall constitute a quorum and the affirmative vote of a majority of those voting at any meeting at which a quorum is present shall constitute action on behalf of the Executive Committee. The Executive Committee shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine.

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by vote of the Board or by law. The Executive Committee may also appoint such committees, including persons who are not Trustees, as it may from time to time approve to make recommendations with respect to matters to be acted upon by the Executive Committee or the Board of Trustees.

(D) The Executive Committee shall keep appropriate minutes of its meetings and its action shall be reported to the Board of Trustees.

(E) The elected Members of the Executive Committee shall constitute a standing "Committee for the Nomination of Officers," responsible for making nominations, at each Annual Meeting of the Corporation, and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk).

XI. A majority of the Trustees, the Executive Committee, or any other committee elected by the Trustees shall constitute a quorum; and a lesser number than a quorum may adjourn any meeting from time to time without further notice. At any meeting of the Trustees, the Executive Committee, or any other committee elected by the Trustees,

the vote of a majority of those present, or such different vote as may be specified by law, the Articles of Organization or these Bylaws, shall be sufficient to take any action.

XII. Any action required or permitted to be taken at any meeting of the Trustees, the Executive Committee or any other committee elected by the Trustees as referred to under Article IX may be taken without a meeting if all of the Trustees or members of such committee, as the case may be, consent to the action in writing and such written consents are filed with the records of meetings. The Trustees or members of the Executive Committee or any other committee appointed by the Trustees may also participate in meeting by means of conference telephone, or otherwise take action in such a manner as may from time to time be permitted by law.

XIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees then in office.

XIV. These Bylaws may be amended by the affirmative vote of the Members at any meeting, provided that notice of the substance of the proposed amendment is stated in the notice of such meeting. As authorized by the Articles of Organization, the Trustees, by a majority of their number then in office, may also make, amend, or repeal these Bylaws, in whole or in part, except with respect to (a) the provisions of these Bylaws governing (i) the removal of Trustees and (ii) the amendment of these Bylaws and (b) any provisions of these Bylaws which by law, the Articles of Organization or these Bylaws, requires action by the Members.

No later than the time of giving notice of the meeting of Members next following the making, amending or repealing by the Trustees of any Bylaw, notice thereof stating the substance of such change shall be given to all Corporation Members entitled to vote on amending the Bylaws.

Any Bylaw adopted by the Trustees may be amended or repealed by the Members entitled to vote on amending the Bylaws.

XV. The account of the Treasurer shall be audited annually by a certified public accountant.

XVI. The Corporation will indemnify every person who is or was a trustee, officer or employee of the Corporation or a person who provides services without compensation to an Employee Benefit Plan maintained by the Corporation, for any liability (including reasonable costs of defense and settlement) arising by reason of any act or omission affecting an Employee Benefit Plan maintained by the Corporation or affecting the participants or beneficiaries of such Plan, including without limitation any damages, civil penalty or excise tax imposed pursuant to the Employee Retirement Income Security Act of 1974; provided, (1) that the Act or omission shall have occurred in the course of the person's service as trustee or officer of the Corporation or within the scope of the employment of an employee of the Corporation or in connection with a service provided without compensation to an Employee Benefit Plan maintained by the Corporation, (2) that the Act or omission be in good faith as determined by the Corporation (whose determination made in good faith and not arbitrarily or capriciously shall be conclusive), and (3) that the Corporation's obligation hereunder shall be offset to the extent of any otherwise applicable insurance coverage, under a policy maintained by the Corporation or any other person, or other source of indemnification.

V. REPORT OF THE DIRECTOR

TO: THE TRUSTEES OF THE MARINE BIOLOGICAL LABORATORY

"We should be careful to get out of an experience only the wisdom that is in it—and stop there; lest we be like the cat that sits down on a hot stove-lid. She will never sit down on a hot stove-lid again—and that is well; but also she will never sit down on a cold one anymore."

—Mark Twain

The calendar year to which this report refers saw many changes. Not the least of these was the departure of James Ebert for the Presidency of the Carnegie Institution of Washington. Ebert's tenure extended through about half of the year, although he was present in person, and by way of generous advice and guidance from Washington in spirit, for a far larger part of it. Other changes have taken place; some in management, some in programs and courses, and some in the Laboratory's rules of operation. A most significant event of the latter kind was adoption by the Trustees, in February of 1979, of a comprehensive seven-year plan for programs, space utilization, and campus improvement.

Many of the transitional changes, like the Trustees' plan and its related actions, came in 1979, and will therefore not be covered in this report. Their occurrence is felt, however, at the time of writing, and their impact upon the style of life and work at the Laboratory will doubtless increase in the year to come.

James D. Ebert

Although a *State of the Institution* report is not the appropriate place for a tribute to its retired chief executive officer, there being better occasions and places, I cannot let pass the opportunity to mark the debt we owe to Dr. Ebert. His long association with the MBL, which we trust will continue, reached a peak of intensity during an influential tenure as the Laboratory's first full-time Director and President. Always forceful and direct in his views, more analytical and better-prepared than most who disagreed with him, Ebert brought about a series of major alterations in the way the MBL functions, meeting successfully, thereby, a number of crises in finance and management. He was able to accomplish this without any infringement of program quality nor of the traditional rights and responsibilities of the Corporation.

I have a more detailed view of his efforts than is generally available, owing to the records of his work, upon which we are daily dependent, and to my now frequent contacts with friends of the Laboratory to whom Ebert first took the MBL's case. There can be no denial of the fact that his efforts were as fruitful as they were tireless and self-sacrificing. I believe that his contribution to the Laboratory will stand permanently as having been central to its survival in strength.

Research

To separate research from education is a process even more artificial for the MBL than it is for universities. The intertwining of the two is here less an accident and more a design than in any other scientific institution. It has been so ever since Director Whitman wrote in the first Annual Report:

"Other things being equal, the investigator is always the best instructor. The highest grade of instruction in any science can only be furnished by one who is thoroughly imbued with the scientific spirit, and who is actually engaged in original work. Whence the propriety—and, I may say, the necessity—of linking the function of instruction with that of investigation."

It is nevertheless useful to report separately upon research activity at the MBL, to the extent that is characterized by numbers of persons so engaged, by the sums awarded and spent for the purpose, and by the facilities given up to it, as opposed, for example, to those employed solely for classroom activities.

We can be proud that in a time of declining real support for research nationwide, and of exponentially-growing costs and demands upon physical facilities, the MBL has been able, upon a comparatively uncertain financial base, to enlarge the scope and impact of its research, and to do so without denying access to its facilities by any properly qualified person or group.

There are four broad areas of research that together encompass most, although not all, investigative activities at the Laboratory. These are (1) Ecology, (2) Cellular, Developmental, and Reproductive Biology, (3) Neuroscience, Physiology, and Biophysics, and (4) Marine Biology and Biomedicine. In each of these areas the Laboratory provides for permanent (or year-round) as well as transient (mainly, but not exclusively summer) programs.

In 1978 there was growth in all four, as measured by people, funds, and—since no decline of standards was, or will ever be permitted—by impact upon biology as a whole, *i.e.*, by quality. The simple argument underlying this last conclusion is from information theory, in which one obtains first the information content per individual in an assemblage (the units being "bits" or "nats," or some other, depending upon the base of logarithms chosen), and then the information content of the entire assemblage by multiplying the result by the number of individuals.

Lest this be interpreted as an argument for unlimited growth, I make haste to demur. It is not. It is, rather, a way of saying that, within limits, growth accompanied by maintained standards is *ipso facto* an increase in total quality.

The Ecosystems Center, the Boston University Marine Program, the Laboratory of Sensory Physiology, and Laboratory of Biophysics, and a group of some twenty individual research programs operated at the MBL have all performed as well or better in 1978 than they did in 1977. By performance I mean the sum of those measures employed in our system of program evaluations: important publications; grants awarded; honors and awards to staff; presentations of important lectures; quality of students and other participants attracted; and the like.

These activities, which comprise the currently permanent part of the MBL's research effort, have already reached a size and level of achievement such that to single out one or two for special mention here would be impolitic. It may well be that we shall soon need a separate Annual Report on Research, in order to do justice to the accomplishments of our permanent programs. Suffice it to say that the health and quality of year-round research at MBL are no longer at issue. The issue will instead be, for the future, to control growth while still retaining a decent flexibility of operation, so that life at MBL does *not* decay to the level of that lived in many universities, where changes in space assignment are accomplished only at the expense of internecine warfare.

In the summer of 1978 research space at the Laboratory was fully occupied, except for a few rooms that fell vacant at a late hour, owing to changes of plans. For the summer of 1979 there were far more applications even than in the prior year. We have been able, at least at the time of writing, to accommodate all those whose proposed research was judged appropriate to our standards by the Research Space Committee. This was accomplished by several devices, including the use for research of rooms not formerly rented for that purpose, and a vigorous effort to encourage the sharing of space, when that would not interfere with research quality. I may say that applicants so approached by us, including Corporation members who have enjoyed a certain luxury in past summers, responded magnificently. No such suggestion for sharing failed to be received courteously and to be considered seriously.

The above is a quantitative statement, of course, about the summer research program, but the argument made earlier about population and information content applies: there is a significant and continuing increase in the demand for research space and facilities here assignable for such use, and the overall quality is rising. All indicators suggest that the trend will continue.

It is evident from the foregoing that we shall have a great deal of decision-making to do in the future as regards deployment of space and facilities. This is a challenge that we ought not to fear: far better to be in a position of making fine distinctions—however fallible peer judgments may sometimes be, and however painful rejection is—than to be unable to assign all the available space for want of good contenders.

A few sample numbers: overhead recovery from non-MBL research grants (via space charges) was \$424,631 in 1977, \$503,576 in 1978, and we have budgeted \$562,699 for 1979. The corresponding figures for MBL-administered grants (government and private) are \$251,916; \$297,136; \$391,766. These number do, of course, have an inflation component, and hence do not imply a *proportionate* increase in the absolute size of the research effort, but they do reflect a real and impressive size increment nevertheless. Were it not that the inflationary rise of operating costs easily matches them, I might be moved to call such figures "healthy."

Some losses and gains of research persons should be mentioned, although of course space limitation prohibits anything like a comprehensive listing. There was the very tragic loss of Dr. Fred Lang, of the B.U.M.P., in an automobile accident, and that must be mentioned. Two other honored and well-known Corporation members died in the course of the year: Jean Clark Dan and Lester G. Barth.

It is a personal pleasure for me and splendid news for the Laboratory that Professor Shinya Inoué, of Pennsylvania, a distinguished cell biologist and long-time Corporation member, will henceforth be in permanent residence at the MBL, and will play an important role in the planning and growth of our cell biology programs. Several additional appointments of importance have been made or are in the making. They will be announced in due course.

Education

As indicated earlier, every research activity at the MBL has a closely-related educational component; hence a mere catalogue of the formal courses does not convey the pervasiveness of learning and instruction that characterizes work here. As is true for research, however, a simple listing can indicate something of the *scope* of the program, and of changes taking place within it, for Corporation members already familiar with it.

Each of the five January courses offered in 1978 was continued in 1979: Behavior (J. Atema); Comparative Pathology of Marine Invertebrates (F. Bang); Developmental Biology (W. Vincent); Ecology (G. Woodwell); and Neurobiology (Alan Fein, together with E. F. MacNichol, Jr. and others, who carried on following the death of Fred Lang). One hundred fourteen students completed courses in the now well-established January semester, to which contributions were made by a group of distinguished teachers from around the nation, as well as from the Woods Hole resident community. The January semester is an obvious success, but its growth in future years will be small, limited by the space and facilities available for it.

Since it is clear that the January semester is to be a recurrent MBL activity, we shall be engaged during months to come in a number of management and quality control initiatives. These will bring the entire program under the same sort of continuous review, eventually, as is applied to the summer courses, possibly by the same committee of the Corporation. We will also attempt to regularize the financial arrangements, i.e.,

those governing tuition charges, reimbursements and compensation for faculty and guest speakers, and the assignment of operating costs among various cost centers of the budget. It is even now clear, however, that the January semester places no great financial burden upon the Laboratory, while yielding large benefits to the community intellectual life during what was formerly a slow month. Of course it provides invaluable educational benefits to the students, whose many letters attesting thereto are a gratifying chapter of our files.

All seven of the distinguished summer courses continued under the same directorates as in 1977, with one hundred forty-eight participants. During the year a number of searches for directorships due to become vacant in 1980 were initiated. Thomas Reese and John Hildebrand have been appointed co-directors of the Neurobiology Course to succeed Edward Kravitz. Outcomes of other searches will be announced in the summer of 1979. The excellent Microbial Ecology program has been elevated, under stimulus of the recommendations of an *ad hoc* committee and with approval of the Standing Committee on Instruction, to the status of a regular MBL summer course. Ably directed in 1978 and in prior years by Holger Jannasch, it will for 1979 and 1980 have Jannasch as co-director with Harlyn Halvorson, who will then continue as Instructor-in-Chief for an additional three years. A major effort is underway to provide this new course with suitable quarters in the Loeb Building by the summer of 1980.

Because of the tragic death of Mrs. Gelperin in May, 1979, Alan Gelperin will be unable to serve, at least for 1979, as director of the course on Neural Systems and Behavior. Faculty member Ronald Hoy agreed promptly and generously to step in as interim director. For this reason we anticipate a smooth operation of the relatively new offering despite the absence, hopefully a temporary one, of its energetic first director.

Active planning was begun in April, 1979, for a 1980 summer course in the Biology of Parasitism. The planning group includes, in addition to a number of MBL administrators and our own Frederik Bang, director of the January course on comparative pathology and a scholar of international reputation in the field, several accomplished members of the Harvard and Rockefeller University faculties, together with scientific officers of the Rockefeller and Edna McConnell Clark Foundations. The MBL Committee on Instruction, also represented among the planners (by Chairman Kaminer), will be concerned with detailed proposals during the summer of 1979.

The spring course in Aquatic Veterinary Medicine, a combined offering of the University of Pennsylvania, Cornell University, and three of the four Woods Hole institutions, directed energetically by Donald Abt, was offered for the second time, and with pronounced success. Interest in the program is sufficiently high to justify not only its continuation, but also expansion of the effort to include post-course research and, possibly, a number of more permanent activities centered at the MBL.

Morton Maser's program of continuing education, in the form of Short Courses, continues to merit the praise accorded it in Dr. Ebert's 1977 report. In 1978 there were eleven such courses, most of them concerned with advanced instrumentation and research technology, and featuring faculties of an exceptional expertise. Among the Instructors-in-Chief were Robert Allen, Blair Bowers, Lee Peachey, Bruce Wetzel, Russell Steere, Eduardo Macagno, Yukata Kobayashi, Patrick O'Farrell, Robert Ivarie, and Maser. A total of 157 participants came to Woods Hole in the months of March, April, May, October, November and December for these high-intensity courses. Their quality has been the subject of uniformly favorable comment, from students and faculty alike. A most enthusiastic article about them has been published in the *Norelco Reporter*.

Funding of all the formal educational offerings is at a high level, although as university science administrators well know, it is impossible to recover the current *real*

costs of advanced laboratory teaching. Nearly every summer course receives direct support from government or private grants, or from a combination of the two. Those few courses that were temporarily without directed support in 1978 have it or are very likely to have it in 1979. With reorganization of the financing and accounting for the January Semester, it will become practical to initiate an energetic program of fund-raising for that venture. Short Courses come very close to paying for themselves; indeed, they are upon a simple cash basis truly self-supporting.

This section cannot close without mention of the Boston University Marine Program, which continues as a leading opportunity, nationally, for graduate students seeking to specialize in marine biology. The MBL's pride in the Program and pleasure in its fine students and faculty is matched by opinion of the faculty and central administration at Boston University. An outstanding new appointment to the B.U.M.P. faculty has been made this year (Dr. Sidney Tamm, a cell biologist who will work with the already prominent MBL motility group). Dr. Christopher H. Price, an active investigator in the field of neurobiology, has been appointed to replace Dr. Lang.

Scientific Meetings and Conferences

Among the blessings that some of us take for granted at the MBL is the skill and efficiency with which housing and other maintenance needs of transient participants in our programs are handled. It is a task of the greatest complexity, involving not only the work of a specialized support staff, but also attention to detail, patience, and tact, coupled with a willingness to deal under pressure with many demands for exceptions to rules. All this Homer Smith does, as he has done for many years, quietly and effectively.

The more remarkable, therefore, is the rapidly increasing exploitation of our Swope Center, comprising as it does a significant additional burden of work. This is done, again, with minimum fuss, and by a support staff that would be viewed as skeletal by the management of a small motel.

In 1978, there were only two months (June and December)—and those with good reason—during which there were *not* significant numbers of working visitors to the MBL, residing in the Swope Center and making use of the Laboratory's facilities for one or another scientific or educational purpose. The total number of such persons reached a remarkable 2,300. Activities and institutions represented included: Massachusetts Marine Educators, St. Mary's College, Purdue University, National Marine Fisheries Service, Yale University, East Coast Nerve Net, International Symposium on the Spermatozoon, M.I.T., the University of Pennsylvania, Harvard University, Drew University, Upsala College, the Society of General Physiologists, the Marine Science Librarians, University of Maryland, Maine Audubon Society, a conference on Biomedical Applications of *Limulus*, Brown University, the New England Estuarine Society, Simmons College, and many others.

The value of such utilization of our facilities, quite aside from the implied financial benefits, is incalculable. All the Woods Hole institutions are beneficiaries (as is the village as a whole), and those perceived values attest to the wisdom of MBL people whose labors and gifts made the Swope Center a reality.

Once again, our problem is now one of choice among worthy competing alternatives, rather than of recruitment of users,—one of growth control, rather than of its simple encouragement. Again, I should be inclined to describe the whole phenomenon as unqualifiedly healthy, were there not the ghost of concern about inexorable inflation of operating costs, which, however carefully *they* are held down by means of economies, tend to alter what should be a surplus-producing function to one that may just pay for itself. The matter of costs and charges in this sphere will receive serious review in the year to come.

Management

Significant changes in management structure and technique will certainly take place in the two or three years ahead, but we have been very cautious about changes during this year of transition. Caution is demanded for more than the usual reasons of morale: most changes in management that *ought* to be made will cost more than current systems, but we are determined that administration shall set an example of cost-consciousness for the institution as a whole.

Francis Bowles, who has served effectively as head of the Department of Research Services, has relinquished that position in favor of an association with the Ecosystems Center, which provides him a deserved increase of opportunity to pursue his own research and technical interests. He has been replaced by Morton Maser, who adds this responsibility to his already major commitment in continuing education and in the operations of service laboratories. In recognition thereof, Maser has been named Assistant Director for Educational and Research Services, with a broad range of defined duties and responsibilities concerned with research support services, equipment space deployment, admissions, and the day-to-day operations of our formal courses. He will work closely with the Director, with the Instructors-in-Chief, and with the Committee on Instruction in an effort to bring an increase of order and predictability into what is an unnecessarily variable system of management.

All activities enumerated in earlier sections need and deserve published notice at a number of levels of specificity—from in-house posting of events schedules to journal articles and press releases. These are the purview of the Public Relations Department which, with minimal staff under the direction of Anne Camille Maher, has met the demand with honor. Among particularly useful ventures of the past year (quite aside from such public events as MBL Day) have been the appearance of a regular and timely calendar of scientific activities, and a new and greatly improved directory of personnel and programs.

Our able Controller, Edward Casey, and his competent staff continue to get things done accurately and on time, and to accomplish that miracle with a staff, space, and machinery that would be more appropriate to an institution half the size of the MBL. Some relief from the burden of work and responsibility will have to be provided for that department soon. In the meantime it is to be commended for its management of accounting and financial control functions. Among those are the duties of Joan Howard, MBL Grants and Contracts Administrator, who deals competently with a volume of government support for research and teaching that would, in better-endowed circumstances, call for an office full of people.

The Library, too, deserves commendation, for the self-effacing but highly professional style with which its services are managed and rendered to the entire community, again under circumstances of fiscal restraint. We will be announcing during the summer of 1979 a number of encouraging initiatives and gifts which, all together, will make easier the work of our Library staff and improve significantly the facilities and services offered to users.

Support

Details of Foundation, Corporate, and other support received during 1978 appear below in a separate listing. For the present it is sufficient to note that Foundation support, specifically, came to \$719,142 in 1978, as compared with \$763,699 in 1977. This is a good showing, considering that it was a year of management transition, requiring a certain temporary slowing of fund-raising activity. In fact gifts received early in calendar year 1979, and others in prospect, make it certain that the two-year totals for 1978 and 1979 will exceed by a large factor those obtained in any prior period of similar

length. This will, it is hoped, be the subject for considerable discussion and action beginning in the late summer of 1979. It is to be noted that the Annual Campaign yielded twice as much support in 1978 as in 1977.

Atlantic Richfield Foundation
 Charles Ulrick & Josephine Bay Foundation
 Charles E. Culpeper Foundation
 Fred Harris Daniels Foundation, Inc.
 Arthur Vining Davis Foundations
 Henry L. and Grace Doherty Charitable Foundation
 Eastern Associated Foundation
 Exxon Corporation
 Foundation for Microbiology
 Walter Henry Freygang Foundation
 Friendship Fund
 General Electric Foundation
 Gillette Charitable & Educational Foundation
 Grace Foundation, Inc.
 Grass Foundation
 Lillia Babbitt Hyde Foundation
 IBM Corporation
 Edward Bangs Kelley & Elza Kelley Foundation
 Henry P. Kendall Foundation
 Charles A. King Trust
 Josiah Macy, Jr. Foundation
 NL Industries
 Jessie Smith Noyes Foundation
 Pfizer, Inc.
 Rockefeller Foundation
 Rowland Foundation, Inc.
 Sandoz Foundation
 Alfred P. Sloan Foundation
 Seth Sprague Educational & Charitable Foundation
 Surdna Foundation
 UPS Foundation

Conclusion

I take the liberty, in closing, of making a personal comment. I hope to be forgiven for it, because the comment is in effect a message to Trustees, but one that ought to be proffered in print.

I have now had a sufficient opportunity to study the MBL and its operations to have some confidence in a judgment of its health. I find the Laboratory in a state of flourishing health; this by comparison with other academic and research institutions with which I have been, and in some cases remain, associated. By health I refer to the *entire* organism; its history, its personality, its prospects, and its physical body. Parts of the body need attention, to be sure, and some growth of the parts is urgent; but that can and will be done. There is every reason to believe that the unique and influential history of the Laboratory is predecessor to a future just as unique and of just as great an influence.

To play a role in fashioning that future, and in solving the important problems that must be solved, is a privilege. To be sure the privilege has to be paid for; the work is as taxing, physically and psychologically, as any I have ever done, and despite every

effort to the contrary I have had to slow somewhat, for a year or so, my efforts in research that I care about a great deal. I am certain now, however, of what I could not have been certain in July of 1978, when I arrived for full-time residence in Woods Hole: the job can be done. It is as important as any job it has ever been my good fortune to work at.

1. THE STAFF

MARINE ECOLOGY

I. INSTRUCTORS

JOHN M. TEAL, Woods Hole Oceanographic Institution, co-director of course
IVAN VALIELA, Boston University Marine Program, co-director of course
CHARLENE VAN RAALTE, Hampshire College

II. ASSISTANTS

MIKE PETROVSKI, Boston University Marine Program
CHRISTOPHER VAN RAALTE, Dalhousie University

III. SPECIAL LECTURERS

ARNE JENSEN, University of Aarhus
THOMAS FENCHEL, University of Aarhus
BRUCE J. PETERSON, Ecosystems Center
JOHN E. HOBBIIE, Ecosystems Center
JAMES W. PORTER, University of Georgia
KAREN G. PORTER, University of Georgia
LAUREN HAURY, Woods Hole Oceanographic Institution
ROBERT PAINE, University of Washington
JOY GEISELMAN, Woods Hole Oceanographic Institution
WENDY WILTSE, Woods Hole Oceanographic Institution
DONALD RHOADS, Yale University
THOMAS JORDAN, Boston University Marine Program
RICHARD HAEDRICH, Woods Hole Oceanographic Institution
JOHN STEELE, Woods Hole Oceanographic Institution
SUSAN PETERSON, Woods Hole Oceanographic Institution
JOHN MASON, Woods Hole Oceanographic Institution
WILLIAM ODUM, University of Virginia
HOWARD SANDERS, Woods Hole Oceanographic Institution
C. A. S. HALL, Cornell University

IV. LECTURES

I. VALIELA	Introduction to course
J. TEAL	Introduction to the oceans
C. VAN RAALTE	Marine ecology and Woods Hole
A. JENSEN	Salt marsh vegetation in Denmark
I. VALIELA	Structure and function of salt marsh ecosystems
A. JENSEN	Phenology and growth of <i>Halimione</i>
B. PETERSON	Techniques and results of phytoplankton production studies
B. PETERSON	The role of plankton in the global carbon cycle
I. VALIELA	Nutrient budget of a salt marsh

T. FENCHEL	Microbial processes in estuarine sediments I
J. HOBBIIE	Bacteria in aquatic systems I
J. HOBBIIE	Bacteria in aquatic systems II
J. PORTER	Life in three dimensions: open ocean vs. terrestrial ecosystems
J. PORTER	Life in two dimensions: substrate-bounded marine ecosystems
J. PORTER	The ecology of marine invertebrate-algal symbioses
K. PORTER	Grazing and planktonic community structure
L. HAURY	Patchiness in Cape Cod Bay
R. PAINE	Plant-herbivore relationship in marine systems
R. PAINE	Disturbance and diversity in the intertidal
J. GEISELMAN	Chemical defenses in marine algae
R. PAINE	Higher order carnivores
W. WILTSE	Effects of the predatory snail, <i>Polinices</i> , on community structure
T. FENCHEL	Microbial processes in estuarine sediments II
C. VAN RAALTE	Nitrogen fixation in salt marshes
D. RHOADS	Infaunal deposit-feeding: climax mud communities
D. RHOADS	Bioturbation and sediment binding
T. JORDAN	Nitrogen budget of mussels
R. HAEDRICH	The nature of deep sea fishes
J. STEELE	Problems with the management of marine resources
S. PETERSON	Changing values in uses of salt marshes
I. VALIELA	Experimental studies of salt marsh pollution
J. MASON	Distribution and behavior of Atlantic blue fin tuna
W. ODUM	The ecology of mangroves
W. ODUM	Detritus feeding in coastal ecosystems
W. ODUM	Venice: a case study
H. SANDERS	The West Falmouth oil spill
C. A. S. HALL	Environmental impact of coastal zone energy facilities
C. A. S. HALL	Models and decision making: Hudson River

EMBRYOLOGY

I. INSTRUCTORS

JOAN V. RUDERMAN, Harvard University, co-director
 TOM HUMPHREYS, University of Hawaii, co-director
 SUSAN V. BRYANT, University of California, Irvine
 GARY L. FREEMAN, University of Texas
 CYRUS LEVINTHAL, Columbia University
 EDUARDO R. MACAGNO, Columbia University

II. CONSULTANTS

R. K. HUNT, Johns Hopkins University
 J. P. TRINKAUS, Yale University
 J. R. WHITTAKER, Wistar Institute

III. ASSISTANTS

CLARISSA CHENEY, University of Pennsylvania
 NIGEL HOLDER, University of California, Irvine
 MARC LAUFER, University of Pennsylvania

IV. LECTURES

G. L. FREEMAN	Localization and the specification of form in development
G. L. FREEMAN	Localization of embryonic determinates in <i>Cerebratulus</i> eggs and early embryos
N. H. VERDOUK	The origin of spatial organization in Molluscan development
M. R. DOHMEN	The relationship between local surface differentiation and cytoplasmic localization
J. R. WHITTAKER	Localization and development in Ascidians eggs and embryos: I and II
L. WOLPERT	Pattern formation in chick limb development
J. P. TRINKAUS	Meroblastic development and gastrulation in <i>Fundulus</i> embryos
J. P. TRINKAUS	Mechanisms of cell movement <i>in vivo</i> during gastrulation
J. V. RUDERMAN	Maternal mRNA and localization
T. HUMPHREYS	The marine sponge, <i>Microciona prolifera</i> and other aggregating cell systems
T. HUMPHREYS	The molecular organization of <i>M. prolifera</i> aggregation factor complex and its active fragments
LAN BO CHEN	Studies on intercellular fibronectin matrices
R. K. HUNT	Specification of positional information in amphibian eye development
R. K. HUNT	Positional information in neural development
C. LEVINTHAL	Three dimensional computer reconstruction of neural anatomy
E. R. MACAGNO	Development of synaptic connections in invertebrate visual systems
C. LEVINTHAL	Development and characterization of identified cells in isogenic fish
S. BRYANT	Pattern regulation in amphibian limb regeneration: I and II
L. JAFFEE	Activation and patterning of animal eggs and oocytes by steady ion currents
S. INOUÉ	Mitosis: Cell structural organization and mechanisms
N. DETERRA	Control of cell division and morphogenesis in <i>Stentor</i>
N. B. GILULA	Gap junctions and communication between cells
N. B. GILULA	Communication in development and differentiation
P. BRYANT	Pattern formation in the imaginal disks of <i>Drosophila</i>
J. SAUNDERS	Positional signaling in chick limb bud development
N. HOLDER	Programmed development of the chick wing
L. ITEN	Pattern regulation in chick limbs
S. KAUFMAN	Control of sequential commitment in <i>Drosophila</i> development
K. KALTHOFF	Analysis of an anterior morphogenetic determinant in the egg of an insect, <i>Smittia sp.</i> , Chironimidae
N. LEDOUARIN	Migration and differentiation of neural crest cells studied in avian embryos with interspecific chimeras
J. ROSENBAUM	Control of flagellar protein synthesis in <i>Chlamydomonas</i>
R. SAGER	Somatic cell genetics: Methodology
R. SAGER	Somatic cell genetics: Application to some fundamental problems in biology
G. STENT	Neuroembryology of the leech

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| Y. PENG LOH | Corticotropin and endorphin peptides. Biosynthesis and their role as intercellular messengers |
| N. MARCUS | Normal and aberrant phenotypes in the developing sea urchin |
| P. GRANT, S. SHARMA,
C. LEVINTHAL, AND
R. K. HUNT | New looks at retinal-tectal connections, a microsymposium |

NEURAL SYSTEMS AND BEHAVIOR

I. INSTRUCTORS

- ALAN GELPERIN, Princeton University, director of course
 JAMES L. GOULD, Princeton University
 ADRIANUS KALMIJN, Woods Hole Oceanographic Institution
 RONALD HOY, Cornell University
 DAVID J. PRIOR, University of Kentucky
 WILLIAM KRISTAN, JR., University of California, San Diego
 RANDOLF MENZEL, Free University, Berlin
 FERNANDO NOTTEBOHM, Rockefeller University

II. ASSISTANTS

- CHRISTINA MYLES, Manchester Community College
 S. REINGOLD, Princeton University

III. VISITING LECTURER

- J. NICHOLLS, Stanford University

IV. SPECIAL LECTURERS

- GEORGE GERSTEIN, University of Pennsylvania
 ROBERT CAPRANICA, Cornell University
 WILLIAM G. QUINN, Princeton University
 ERIC R. KANDEL, Columbia University
 LARRY COHEN, Yale University
 TOM EISNER, Cornell University
 DONALD GRIFFIN, Rockefeller University
 MICHAEL V. L. BENNETT, Albert Einstein University
 EDUARDO MACAGNO, Columbia University
 GUNTHER STENT, University of California, Berkeley

V. LECTURES

- | | |
|------------|--|
| J. GOULD | Introduction to classical ethology |
| J. GOULD | Neuroethology of <i>E. coli</i> |
| J. GOULD | Communication in honey bees |
| J. GOULD | Orientation and navigation in honey bees |
| A. KALMIJN | Electroreception in fish |
| A. KALMIJN | Active and passive electro-orientation |
| A. KALMIJN | Physics and physiology of electroreception |
| A. KALMIJN | Magnetic orientation |
| R. HOY | Genetics and neurobiology of cricket song |

R. HOY	Acoustic interneurons
R. HOY	Specificity and mechanisms of neural regeneration
D. PRIOR	Temperature acclimation in neural systems
D. PRIOR	Behavioral switching
D. PRIOR	Environmental modulation of reflex pathways
J. NICHOLLS	The leech: Sensory and motor neurons
J. NICHOLLS	The leech: After-effects of activity
J. NICHOLLS	The leech: Chemical and electrical transmission and quantal analysis
B. KRISTAN	Pattern generation
B. KRISTAN	Leech heartbeat control system
B. KRISTAN	Leech swimming I
B. KRISTAN	Leech swimming II
A. GELPERIN	Neuroethology of molluscan learning
A. GELPERIN	Serotonin and synaptic modulation
A. GELPERIN	Comparative physiology of feeding
R. MENZEL	Color vision in invertebrates
R. MENZEL	Wavelength selective behavior
R. MENZEL	Physiology of insect learning
T. EISNER	Herbivore-plant interactions II
F. NOTTEBOHM	Selective learning
F. NOTTEBOHM	Evolution of vocal learning
F. NOTTEBOHM	Origins and mechanisms of hemispheric dominance
F. NOTTEBOHM	Birdsong, a neuroethological model

EXPERIMENTAL MARINE BOTANY

(COMPARATIVE BIOLOGY AND BIOCHEMISTRY OF ALGAE)

I. INSTRUCTORS

JEROME A. SCHIFF, Brandeis University, director of course
 JAMES FIORE, Suffolk University, laboratory instructor
 HARVARD LYMAN, State University of New York at Stony Brook, laboratory instructor
 DAVID MAUZERALL, Rockefeller University
 ROBERT TROXLER, Boston University

II. CONSULTANTS

ROBERT L. GUILLARD, Woods Hole Oceanographic Institution
 FRANK A. LOEWUS, Washington State University
 RALPH S. QUATRANO, Oregon State University

III. STAFF ASSOCIATES

SEYMOUR COHEN, State University of New York at Stony Brook
 CATHERINE FUSSEL, Pennsylvania State University
 HANS GAFFRON, Woods Hole, Massachusetts and Sanibel, Florida
 ANDREW HOLOWINSKY, Brown University

IV. ASSISTANTS

JOANNE LUEBBERT, State University of New York at Stony Brook
 SCOTT SCHATZ, University of Rhode Island
 SHARON SUBYAK, Suffolk University

V. SPECIAL LECTURERS

VERNON AHMADJIAN, Clark University
 MARY M. ALLEN, Wellesley College
 MARTHA BERLINER, Simmons College
 ANNETTE COLEMAN, Brown University
 FRANKLIN FONG, Texas A & M University
 JANE GIBSON, Cornell University
 ROBERT L. GUILLARD, Woods Hole Oceanographic Institution
 PETER HEPLER, University of Massachusetts
 PETER HEYWOOD, Brown University
 LIONEL JAFFE, Purdue University
 ALFRED LOEBLICH, Harvard University
 LYNN MARGULIS, Boston University
 EYTANA PADAN, Hadassah Medical School
 CARL PRICE, Rutgers University
 JOEL ROSENBAUM, Yale University
 WOLFHARDT RÜDIGER, University of Munich
 GREGORY SCHMIDT, Rockefeller University
 JAMES R. SEARS, Southeastern Massachusetts University
 RAYMOND E. STEPHENS, Marine Biological Laboratory
 ARTHUR I. STERN, University of Massachusetts
 STAN WATSON, Woods Hole Oceanographic Institution
 ROBERT WILCE, University of Massachusetts
 C. L. F. WOODCOCK, University of Massachusetts

VI. LECTURES

J. A. SCHIFF	Chemical Phase of Evolution; biogeochemistry
J. A. SCHIFF	Appearance of Oxygen
J. A. SCHIFF	Evolution of Procarvates
J. A. SCHIFF	Evolution of Eucaryotes & Organelles
J. A. SCHIFF	Evolution of Life Cycles
J. A. SCHIFF	Nutritional Cycles
J. A. SCHIFF	Metabolism of Nitrogen and Sulfur
L. MARGULIS	Microbial Evolution
C. L. F. WOODCOCK	Biology of <i>Acetabularia</i>
R. TROXLER	Biosynthesis of Heme and Chlorophyll, Early Stages
R. TROXLER	Biosynthesis of Heme and Chlorophyll, Later Stages
R. TROXLER	Open Chain Tetrapyrroles
D. MAUZERALL	Photochemical Principles
D. MAUZERALL	Photochemistry of Photosynthesis
J. GIBSON	Photosynthetic Electron Transport
A. STERN	Photophosphorylation
L. JAFFE	Development of Polarity
J. ROSENBAUM	Control of Flagellar Development in <i>Chlamydomonas</i>
R. E. STEPHENS	Microtubules in Plant Development
A. HOLOWINSKY	Wall Synthesis, Deformation and Morphogenesis in Algae
F. FONG	Phototaxis in <i>Euglena</i>
J. SEARS	Vertical Distribution of Algae
R. WILCE	Phytogeographic Studies in the North Atlantic
J. FIORE	Life Histories of the Brown Algae
P. HEPLER	Stomatal Mechanisms
M. M. ALLEN	Biology of Blue Green Algae (Cyanobacteria) I

S. COHEN	Polyamines
J. A. SCHIFF	Chloroplast Development I
H. LYMAN	Chloroplast Development II and III
A. HOLOWINSKY	Circadian Rhythms
H. LYMAN	Quantitative Measurement of Cell Constituents
H. LYMAN	Pigment Methodology
H. LYMAN	Xanthophyta, Chrysophyta, Bacillariophyta
A. LOEBLICH	Unique Cytology and Molecular Biology of Dinoflagellates
J. A. SCHIFF/H. LYMAN	Euglenophyta and Cryptophyta
P. HEYWOOD	Chloromonads
H. LYMAN	Chlorophyta I: Unicells
J. A. SCHIFF	Development of Single Cells from <i>Prasiola</i>
A. COLEMAN	Chlorophyta II: Biology of the Colonial Green Flagellates
J. FIORE	Field and Preservation Methods
R. GUILLARD	Phytoplankton Ecology I and Ecology II
J. FIORE	Chlorophyta III, Chlorophyta IV and Chlorophyta V
C. PRICE	Principles Affecting the Isolation of Cells and Subcellular Structures
J. FIORE	Pheophyta I, II, and III
J. FIORE	Rhodophyta I, II and III
J. FIORE	Distribution of Eastern Macroalgae
A. HOLOWINSKY	Phytochrome-Controlled Responses
STAN WATSON	Procaryotic Membranes
M. M. ALLEN	Biology of Blue Green Algae (Cyanobacteria) II
S. COHEN	Cyanophages
G. SCHMIDT	<i>In Vitro</i> Synthesis and Assembly of Chloroplast Proteins
V. AHMADJIAN	Experimental Studies of the Lichen Symbiosis
M. BERLINER	Protoplasts of Unicellular Green Algae
H. LYMAN	Algal Symbioses
E. PADAN	Facultative anoxygenic photosynthesis in blue-green algae
W. RÜDIGER	Studies of phytochrome and chlorophyll synthesis

NEUROBIOLOGY

I. INSTRUCTORS

EDWARD A. KRAVITZ, Harvard Medical School, director of course
 JONATHAN B. COHEN, Harvard Medical School
 EDWARD J. FURSHPAN, Harvard Medical School
 DAVID D. POTTER, Harvard Medical School
 JOHN G. HILDEBRAND, Harvard Medical School
 GERALD D. FISCHBACH, Harvard Medical School
 PETER R. MACLEISH, Harvard Medical School
 STORY C. LANDIS, Harvard Medical School
 JOHN HEUSER, University of California, San Francisco Medical School
 PAUL O'LAGUE, University of California, Los Angeles
 THOMAS S. REESE, National Institutes of Health

II. STAFF ASSOCIATES

S. MATSUMOTO, Harvard Medical School
 B. BATTELLE, National Institutes of Health
 R. HARRIS-WARRICK, Harvard Medical School
 M. NELSON, Harvard Medical School

S. GLUSMAN, Harvard Medical School
 D. LANDIS, Harvard Medical School
 M. RHEUBEN, Penn State University

III. ASSISTANTS

SUSAN HUTTNER, University of California, Los Angeles
 J. LAFRATTA, Harvard Medical School
 RUTH SIEGEL, Harvard Medical School
 R. NEUBIG, Harvard Medical School
 B. REESE, National Institutes of Health

IV. SYMPOSIA AND SEMINARS

Symposium I Slow Physiological Events

S. W. KUFFLER, Harvard Medical School
 R. W. TSIEN, Yale University
 E. R. KANDEL, Columbia University

Symposium II Selectivity

D. PURVES, Washington University
 B. G. WALLACE, Stanford University
 U. J. McMAHAN, Stanford University
 J. DIAMOND, McMaster University

Special Saturday Seminars

C. ARMSTRONG, University of Pennsylvania
 T. N. WIESEL, Harvard Medical School
 R. RAHAMIMOFF, Hebrew University, Israel

Regular Seminar Series

D. D. POTTER, Harvard Medical School
 J. BROWN, State University of New York at Stony Brook
 A. E. STUART, Harvard Medical School
 T. S. REESE, National Institutes of Health
 R. LLINAS, New York University
 D. GOODENOUGH, Harvard Medical School
 L. JAN, Y. N. JAN, Harvard Medical School
 E. MACAGNO, Columbia University
 M. GUTHRIE, Commission to Combat Huntington's Disease
 R. MURPHY, State University of New York Albany
 J. CARTAUD, Paris
 H. POLLARD, National Institutes of Health
 R. HOY, Cornell University
 T. SEELEY, Harvard University
 J. KIRZ, State University of New York at Stony Brook

V. LECTURES

J. HEUSER	Structural features of nervous systems
S. LANDIS	Presynaptic structure

S. LANDIS	Postsynaptic structure
T. REESE	Growth cones and synaptic development
G. FISCHBACH	Myogenesis and development of chemosensitivity
G. FISCHBACH	Neurogenesis (neural tube) and development of electrical excitability
N. LEDOURAIN	The neural crest
P. MACLEISH	Synapse formation: I. Early stages of transmitter release
G. FISCHBACH	Synapse formation: II. Organization of the postsynaptic membrane
J. COHEN	Subcellular fractionation of Torpedo electric organ
R. NEUBIG	Identification of neurotransmitter receptors by ligand binding
J. COHEN	Biochemical characterization of the nicotinic cholinergic receptor
J. CARTAUD	Morphological studies of nicotinic cholinergic receptors and acetylcholinesterase
J. COHEN	Neurotransmitter receptors and their responses, an overview
J. HILDEBRAND	Introduction. Acetylcholine
J. HILDEBRAND	Catecholamines
E. KRAVITZ	GABA
M. NELSON	Neuroethology
E. KRAVITZ	Amines and Modulation
B. BATTELLE	Cyclic AMP
E. KRAVITZ	Peptides

PHYSIOLOGY

I. INSTRUCTORS

K. E. VAN HOLDE, Oregon State University, director of course
 MARK MOOSEKER, Yale University
 RAY STEPHENS, Marine Biological Laboratory
 JAY BROWN, University of Virginia
 JOHN WOOLEY, Princeton University
 TOM BALDWIN, University of Illinois
 F. DAHLQUIST, University of Oregon
 SUSAN ASTRIN, Institute of Cancer Research

II. CONSULTANTS

RUTH SAGER, Sidney Farber Institute for Cancer Research
 ALEX RICH, Massachusetts Institute of Technology

III. STAFF ASSOCIATES

AL CHRISTOPHER, University of Chicago
 MIRIAM BALDWIN, University of Illinois
 W. DENTLER, University of Kansas
 JERRE SUMMERS, Cancer Research Institute
 K. TATCHELL, Oregon State University

IV. ASSISTANTS

PAT GALVIN, University of Denver
 DAVID VONHIPPEL, University of Oregon

V. SPECIAL LECTURERS

PHIL BASSFORD, Harvard Medical School
 SU CHUNG, Harvard University
 NOEL DE TERRA, Haneman Hospital
 H. EISENBERG, Weizmann Institute, Israel
 SARAH ELGIN, Harvard University
 R. GENNIS, University of Illinois
 N. B. GILULA, The Rockefeller University
 ALFRED GOLDBERG, Harvard Medical School
 STEVE HARRISON, Harvard University
 RHU CHI HUANG, John Hopkins University
 S. INOUÉ, University of Pennsylvania
 I. ISENBERG, Oregon State University
 ROBERT JACKSON, The Rockefeller University
 NICOLE LE DOUARIN, Marine Biological Laboratory
 ALLAN MAXAM, Harvard University
 T. POLLARD, Johns Hopkins University
 HANS RIS, University of Wisconsin
 JOEL ROSENBAUM, Yale University
 PETER SETLOW, University of Connecticut Health Center
 ALBERT SZENT-GYÖRGII, Marine Biological Laboratory
 ANDREW SZENT-GYÖRGII, Brandeis University
 L. TILNEY, University of Pennsylvania
 ANNA MARIE WEBER, University of Pennsylvania

VI. LECTURES

K. E. VAN HOLDE	Introduction to chromatin structure: Evidence from nuclease digestion and hydrodynamic studies
J. WOOLEY	Introduction to chromatin structure: evidence from electron microscopy and scattering studies
T. BALDWIN	Bioluminescence and bacterial luciferase
ANDREW SZENT-GYÖRGII	Introduction to contractile proteins
A. M. WEBER	Properties of the actin filament
M. MOOSEKER	The control of actomyosin-mediated motility in non-muscle cells
L. TILNEY	The role of actin in non-muscle motility
M. MOOSEKER	Actin-membrane association
R. STEPHENS	Microtubules, I and II
A. MAXAM	Nucleic acid chemistry and DNA sequences
W. DENTLER	Microtubule/membrane interaction in cilia and flagella
K. E. VAN HOLDE	DNA-protein interactions in the nucleosome
S. CHUNG	Characterization of the histone core complex
J. WOOLEY	Molecular architecture of the nucleosome
S. ELGIN	Chromosomal structure and function in <i>Drosophila</i>
I. ISENBERG	Histone-histone interaction
H. RIS	Higher order structure in chromosomes
J. BROWN	The fluid mosaic model of membrane structures
R. GENNIS	<i>E. coli</i> pyruvate oxidase—studies on lipid-protein interactions
T. WEGMANN	Structure and function of histocompatibility antigen on a membrane protein

P. BASSFORD	Transport and secretion in gram-negative bacteria
R. DAHLQUIST	Lipid-protein interactions
R. DAHLQUIST	Bacterial chemotaxis, a model sensing system
S. INOUE	Mitosis cell structural organization and mechanisms
N. DETERRA	Control of cell division and morphogenesis in <i>Stentor</i>
N. B. GILULA	Gap junctions and communication between cells
N. B. GILULA	Communication in development and differentiation
H. EISENBERG	Protein-DNA interactions
S. ASTRIN	Control of transcription in eukaryots
S. ASTRIN	Control of endogenous viral gene expression in chick embryos
J. BROWN	Protein components of the L-cell plasma membrane
A. GOLDBERG	Studies on the mechanism of protein breakdown in animal and bacterial cells
T. BALDWIN	Role of proteases in controlling enzyme levels
R. JACKSON	Signal peptidase and its role in the transfer of polypeptides across the RER membranes
P. SETLOW	Protein regulation during bacterial spore germination
R. C. HUANG	Initiation of RNA synthesis <i>in vitro</i>
N. LEDOUARIN	Migration and differentiation of neural crest cells studied in avian embryos with interspecific chimeras
J. ROSENBAUM	Control of flagellar protein synthesis in chimeras
R. SAGER	Somatic cell genetics I. methodology
R. SAGER	Somatic cell genetics II. Application to some fundamental problems in biology
S. HARRISON	Protein flexibility and macromolecular assembly: virus structures at high resolution
A. RICH	Molecular structure and biological function of t-RNA
ALBERT SZENT-GYÖRGHI	The living state

RESEARCH PROGRAM IN MICROBIAL ECOLOGY

I. INSTRUCTORS

JANE A. GIBSON, Cornell University
 ROBERT E. HUNGATE, University of California, Davis
 HOLGER W. JANNASCH, Woods Hole Oceanographic Institution
 ALEX KEYNAN, The Hebrew University, Jerusalem
 EDWARD R. LEADBETTER, University of Connecticut, Storrs
 JEANNE S. POINDEXTER, Public Health Research Institute, New York

II. CONSULTANTS

HARLYN O. HALVORSON, Brandeis University
 J. WOODLAND HASTINGS, Harvard University
 ROGER Y. STANIER, Institut Pasteur, Paris
 EDWARD O. WILSON, Harvard University

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 MARK A. SCHNEIDER, Amherst College
 CRAIG D. TAYLOR, Woods Hole Oceanographic Institution

IV. ASSISTANT

STEPHEN J. GIOVANNONI, Boston University

V. SPECIAL LECTURERS

RICHARD P. BLAKEMORE, University of New Hampshire, Durham
 ALASDAIR COOK, Cornell University
 BERNARD D. DAVIS, Harvard University
 ARNOLD L. DEMAIN, Massachusetts Institute of Technology
 IAN DUNDAS, University of Bergen, Norway
 JOHN W. FARRINGTON, Woods Hole Oceanographic Institution
 TOM FENCHEL, University of Aarhus, Denmark
 JAMES G. FERRY, Virginia Polytechnical Institute, Blacksburg
 JOEL C. GOLDMAN, Woods Hole Oceanographic Institution
 EVERETT P. GREENBERG, Harvard University
 HARLYN O. HALVORSON, Brandeis University
 J. WOODLAND HASTINGS, Harvard University
 ETANA PADAN, The Hebrew University, Jerusalem
 ANTONIO H. ROMANO, University of Connecticut, Storrs
 E. G. RUBY, Harvard University
 IVAN VALIELA, Marine Biological Laboratory
 JOHN B. WATERBURY, Woods Hole Oceanographic Institution

VI. LECTURES

H. W. JANNASCH	Introduction to microbial ecology I and II
H. W. JANNASCH	Continuous culture of microorganisms
H. W. JANNASCH	Continuous culture in microbial ecology
H. W. JANNASCH	Practice of the chemostat
H. W. JANNASCH	Experiments in deep sea microbiology
J. A. GIBSON	The photosynthetic bacteria I and II
J. A. GIBSON	Uptake measurements in microbial ecology
J. A. GIBSON	Nutrient uptake in cyanobacteria
J. A. GIBSON	Survival of prokaryotes: envelope changes
E. R. LEADBETTER	Ways of making a living: anaerobes
E. R. LEADBETTER	Ways of making a living: aerobes
E. R. LEADBETTER	Microbial attack on hydrocarbons
E. R. LEADBETTER	Survival of prokaryotes: ecological aspects
E. R. LEADBETTER	Microbiology of the tooth surface
R. E. HUNGATE	Analysis of a microbial ecosystem
R. E. HUNGATE	Cultivation of anaerobes
R. E. HUNGATE	The rumen as an ecosystem I and II
J. S. POINDEXTER	The bacterial prostheca: occurrence, structure and possible function
J. S. POINDEXTER	The bacterial prostheca: developmental studies
J. S. POINDEXTER	Survival of prokaryotes: the vegetative cell
A. KEYNAN	Introduction to the light emitting bacteria II
A. KEYNAN	Experimentation with bacterial bioluminescence
A. KEYNAN	Survival of prokaryotes: the endospore
C. D. TAYLOR	The biology of methane formation
C. D. TAYLOR	The ecology of methane formation
C. D. TAYLOR	The effect of pressure on bacterial growth
R. L. CUHEL	Psychrophilic bacteria

R. P. BLAKEMORE	Magnetotactic bacteria
B. D. DAVIS	Mechanism of protein secretion across membranes
A. COOK	Microbial degradation of pesticides
A. L. DEMAİN	Do antibiotics have a function in natural populations of microorganisms?
I. DUNDAS	Halophilic bacteria
J. W. FARRINGTON	Biochemistry of methane in oceanic environments
T. FENCHEL	Microbial transformations in sediment I and II
J. G. FERRY	The biology of sulfate reduction
J. G. FERRY	Sulfate reduction in marshes and estuaries
J. C. GOLDMAN	Continuous culture of algae
E. P. GREENBERG	The Spirochaetes
H. O. HALVORSON	Survival of prokaryotes: molecular aspects
J. W. HASTINGS	Introduction to the light emitting bacteria I
E. PADAN	Anoxic photosynthesis in cyanobacteria
A. H. ROMANO	The biology of Sphaerotilus
E. G. RUBY	Taxonomy and distribution of luminous bacteria
I. VALIELA	The Sippewisset marsh
J. B. WATERBURY	Marine cyanobacteria

YEAR-ROUND PROGRAMS

1978

BOSTON UNIVERSITY MARINE PROGRAM

THE STAFF

ARTHUR HUMES, Boston University Marine Program, director
 JELLE ATEMA, Boston University Marine Program
 BJÖRN GANNING, University of Stockholm, Sweden
 STJEPKO GOLUBIC, Boston University
 C. K. GOVIND, University of Toronto
 FREDERICK LANG, Boston University Marine Program
 CHARLES LENT, Brown University
 IVAN VALIELA, Boston University

THE ECOSYSTEMS CENTER

GEORGE M. WOODWELL, director

JOHN E. HOBBIÉ, senior scientist; DANIEL B. BOTKIN, associate scientist; FRANCIS P. BOWLES, JERRY M. MELILLO, BRUCE J. PETERSON, assistant scientists; RICHARD H. BURROUGHS, senior fellow; JOHN T. FINN, postdoctoral fellow; ERENE V. PECAN, assistant to the director and research associate; MARY LOUISE MONTGOMERY, assistant to the director; RICHARD A. HOUGHTON, PAUL A. STEUDLER, research associates; WILLIAM J. BEHRENS, JOANNE CLARK, COLLEEN M. CAVANAUGH, KEITH N. ESHLEMAN, JOHN V. HELFRICH, VOYTEK KIJOWSKI, FREDERIC LIPSCHULTZ, KATHERINE C. PARSONS, DAVID S. SCHIMEL, JEFFREY B. SHELKEY, HEDY SLADOVICH, research assistants; PAUL DETWILER, graduate research fellow; JAMES T. MORRIS, WILLIAM B. BOWDEN, JAMES P. REED, graduate research assistants; MICHELLE DIONNE, graduate assistant; GREGG DIONNE, LAWRENCE HOBBIÉ, ANN LEWANDOWSKI, ANDREA R. TURNER, laboratory assistants; MARK WHITE, technician; JOAN M. UPTON, secretary; NANCY L. CAMPBELL, JEANNE FERRARI, FRANCES A. SEYMORE, typists.

JANUARY COURSES 1978

BEHAVIOR

*(Offered Jointly by Boston University Marine Program and the
Marine Biological Laboratory)*

I. INSTRUCTORS

JELLE ATEMA, Boston University, director of course
ADRIANUS KALMIJN, Woods Hole Oceanographic Institute
TERRY CROW, Marine Biological Laboratory
TONY SWAIN, Boston University
VINCENT DETHIER, University of Massachusetts
ROBERT BARLOW, Syracuse University
MARGARET NELSON, Harvard University
BEHRUS JAHAN-PARWAR, The Worcester Institute
IZJA LEDERHENDLER, Marine Biological Laboratory
DANIEL STENZLER, Boston University
LEHR BRISBIN, University of Georgia
JOHN PALMER, University of Massachusetts
MEL KREITHEN, Cornell University
ELIZABETH RUSSELL, The Jackson Laboratory
TIMOTHY WILLIAMS, Swarthmore College
DOUGLAS S. RIGGS, Hampshire College
WILLIAM WATKINS, Woods Hole Oceanographic Institute
KATHY PAYNE, The Rockefeller University
BERNT WÜRSIG, State University of New York at Stony Brook
JANE FRICK, Woods Hole, Massachusetts
DIETLAND MÜLLER-SCHWARZE, State University of New York College of Environmental
Science and Forestry
ARTHUR SILVERSTEIN, Johns Hopkins School of Medicine
EMIL MENZEL, State University of New York at Stony Brook
BORI OLLA, National Marine Fisheries Service
ALASTAIR STUART, University of Massachusetts at Amherst
GEORGE MICHEL, Boston University
FRANCIS BOWLES, Marine Biological Laboratory
STUART MACKAY, Boston University
DANIEL ALKON, Marine Biological Laboratory

II. LECTURES

J. ATEMA	Introduction
J. ATEMA	Sensory physiology and behavior
J. ATEMA	Chemo- and mechanoreceptors
A. KALMIJN	Electroreception with and without electric organs
J. ATEMA	Evolution of chemical senses
A. KALMIJN	Electroreception: the detection of inanimate electric fields
A. KALMIJN	The physics and physiology of electroreception
T. CROW	Statistical considerations in the design of behavioral experiments
A. KALMIJN	Electroreception: more questions with or without answers
V. DETHIER	Insect taste: Food discrimination in plant-feeding insects
T. SWAIN	Chemical compounds in plants affecting insect herbivores
V. DETHIER	What chemosensory neurons tell the brain

R. BARLOW	The eye and the brain
R. BARLOW	Vision and <i>Limulus</i> : a multidisciplinary analysis
M. NELSON	Introduction to neural control of behavior
M. NELSON	Hissing and social behavior in cockroaches
B. JAHAN-PARWAR	Chemosensory behavior in the seahare, <i>Aplysia</i>
I. LEDERHENDLER	Social behavior in <i>Aplysia</i>
D. STENZLER	The burial alarm response and olfaction in the mud snail
L. BRISBIN	Ecology, domestication and the behavior of dogs: some principles and their application to present-day problems of men, wolves, and dogs
L. BRISBIN	The domestic dog today: Training and tracking
J. ATEMA	Catfish social behavior data analysis
J. PALMER	Introduction to biological rhythms
J. PALMER	Biological rhythms in shore dwelling animals
J. PALMER	Human rhythms
M. KREITHEN	Introduction to bird migration
M. KREITHEN	Orientation and infra-sound
T. WILLIAMS	Pleiotropism and the nature of the W-Locus in the mouse
D. RIGGS	Selected topics in biomathematics
W. WATKINS	Sperm whale acoustic behavior
K. PAYNE	Annual change in songs of Humpback whales
B. WÜRSIG	Group composition and stability of coastal bottlenose porpoises
D. SMITH	Introduction to social behavior in birds
D. SMITH	Acoustic and visual signals in redwing blackbirds
J. FRICK	Migration of green turtles
D. MÜLLER-SCHWARZE	Introduction to pheromones
D. MÜLLER-SCHWARZE	Mammalian pheromones
D. MÜLLER-SCHWARZE	Predator-prey relations in Antarctic bird communities
D. MÜLLER-SCHWARZE	Ecology of the reindeer culture
A. SILVERSTEIN	The generation of immunologic diversity: Phylogenists vs. Ontogenists
E. MENZEL	Social organization in Chimpanzees
E. MENZEL	Social organization in Macaques
E. MENZEL	Primate intelligence
B. OLLA	Social behavior and rhythms in marine fishes as related to environmental factors
A. STUART	Social behavior of insects
A. STUART	Termite communication and social behavior
J. ATEMA	Social behavior and pheromones in lobsters
G. MICHEL	The European Ethologists as seen by an American Psychologist
J. ATEMA	Behavior assays in pollution research
F. BOWLES	Territories and social behavior of lobster fishermen
S. MACKAY	Bio-medical telemetry in behavioral studies
D. ALKON	Associative training in a nudibranch mollusc <i>Hermisenda</i>
D. ALKON	Neural substrates of associative training in <i>Hermisenda</i>

DEVELOPMENTAL BIOLOGY

I. INSTRUCTORS

WALTER S. VINCENT, University of Delaware, director of course
 NANCY H. MARCUS, Woods Hole Oceanographic Institution

JAMES D. EBERT, Marine Biological Laboratory
 KENNETH T. EDDS, Marine Biological Laboratory
 SUSAN GERBI, Brown University
 SHINYA INOUÉ, University of Pennsylvania
 WILLIAM MASSEVER, Brown University
 RICHARD MILLER, Temple University
 RAYMOND E. STEPHENS, Marine Biological Laboratory

II. ASSISTANT

DAVID MOSS, University of Delaware

III. SPECIAL LECTURERS

EUGENE BELL, Massachusetts Institute of Technology
 DAVID FRANCIS, University of Delaware
 HARLYN HALVORSON, Brandeis University
 ARTHUR HUMES, Boston University Marine Program
 HANS LAUFFER, University of Connecticut
 DOLORES SCHENDEL, Sloan-Kettering Institute
 RICHARD TASCA, University of Delaware

IV. LECTURES

W. S. VINCENT	Introduction to course
W. S. VINCENT	Gametogenesis I, II, and III
N. H. MARCUS	Differentiation and development of sea urchins in laboratory culture I and II
W. MASSEVER	Comparative oogenesis I
W. MASSEVER	Maturation and ovulation I and II
W. MASSEVER	Vitellogenesis
S. INOUÉ	Spicule development in sea urchins I and II
S. INOUÉ	Techniques of phase and polarizing microscopy
S. INOUÉ	Experimental analysis of spindle structure I
S. INOUÉ	Experimental analysis of mitosis
J. D. EBERT	Ionic regulation of embryonic induction, differentiation in growth
R. MILLER	Gamete activation I and II
W. S. VINCENT	Principles and practices of cell fractionation and gradient analysis I
R. MILLER	Fertilization I and II
M. D. MASER	Techniques and demonstration of electron microscopy
K. T. EDDS	Cleavage and control of cell divisions I and II
W. S. VINCENT	Principals and practice of gradient analysis II
E. RUSSELL	Pleiotropism and the nature of the W-locus in mice
K. T. EDDS	The role of microfilaments in cell division
W. S. VINCENT	Processing of eucaryotic transcripts
W. S. VINCENT	Sequence conservation in ribosomal RNA
S. GERBI	DNA sequence organization on eucaryotic genomes I and II
H. LAUFFER	Analysis of gene activity in <i>Chironomus</i> development
H. LAUFFER	Specific message isolation and identification in <i>Chironomus</i>
J. COLLIER	Normal development of Spiralia

J. COLLIER	Experimental analysis of the development of spiralian
J. COLLIER	Molecular biology of spiralian development I and II
E. ANDERSON	Early embryogenesis in mammals
A. SILVERSTEIN	The generation of immunologic diversity: phylogenists <i>vs.</i> ontogenists
R. E. STEPHENS	Chemistry and structure of microtubules
R. E. STEPHENS	Morphogenesis of cilia
H. O. HALVORSON	New techniques in approaching developmental problems
J. D. EBERT	Birth defects: Biological and ethical considerations
W. S. VINCENT	The politics of the recombinant DNA controversy
N. H. MARCUS	Phenotypic plasticity in marine invertebrates
ALBERT SZENT-GYÖRGYI	Protein, ascorbic acid and cancer

ECOLOGY

I. INSTRUCTORS

GEORGE M. WOODWELL	The Ecosystems Center, director of course
DANIEL B. BOTKIN	The Ecosystems Center
JOHN E. HOBBIE	The Ecosystems Center
JERRY M. MELILLO	The Ecosystems Center

II. SPECIAL LECTURERS

S. H. BERWICK	Yale
KENNETH O. EMERY	Woods Hole Oceanographic Institution
T. FENCHEL	University of Aarhus, Denmark
C. A. S. HALL	Cornell University
HOLGER W. JANNASCH	Woods Hole Oceanographic Institution
MARILYN J. JORDAN	The Ecosystems Center
THOMAS E. LOVEJOY	World Wildlife Fund
BRUCE J. PETERSON	The Ecosystems Center
G. ROWE	Woods Hole Oceanographic Institution
J. H. RYTER	Woods Hole Oceanographic Institution
HOWARD L. SANDERS	Woods Hole Oceanographic Institution
LAWRENCE B. SLOBODKIN	State University of New York at Stony Brook
FREDERICK E. SMITH	Harvard University
J. STEELE	Woods Hole Oceanographic Institution
IVAN VALIELA	Boston University

III. LECTURES

G. M. WOODWELL	Structure of the biosphere I and II
H. SANDERS	Natural communities and evolutionary strategies I and II
L. SLOBODKIN	Evolution: The development of species
L. SLOBODKIN	Group selection: The development of communities
K. O. EMERY	Geological factors: Continental drift
G. M. WOODWELL	Climatic factors: The vegetation of the earth
G. ROWE	The oceans: Distribution of primary production
G. ROWE	The oceans: Secondary production
G. M. WOODWELL	Primary production and the metabolism of the earth
G. M. WOODWELL	The world carbon budget: The predominance of forests
J. E. HOBBIE	The world carbon budget: The role of oceans
J. E. HOBBIE	Primary productivity in aquatic systems I and II

C. A. S. HALL	Secondary productivity I and II
S. H. BERWICK	Biogeography I and II
D. B. BOTKIN	Succession and stability I and II
G. M. WOODWELL	Nutrients and communities I and II
H. W. JANNASCH	The microbiology of S-transformations I and II
B. J. PETERSON	Nutrient limitation in aquatic ecosystems
B. J. PETERSON	Phosphorus cycle of the ocean
J. M. MELILLO	Nutrient cycling in forested basins I and II
I. VALIELA	Nutrient cycling in a salt marsh
G. M. WOODWELL	Ecosystems, energy and world politics
T. E. LOVEJOY	Endangered species
J. H. RYTHER	Oceanic productivity
J. STEELE	The North Sea fishery I and II
S. B. PETERSON	Northwest Atlantic fishery I and II
K. O. EMERY	Mineral resources of the deep sea and international politics
F. E. SMITH	Urban ecology I and II
M. J. JORDAN	Ecological effects of toxins
G. M. WOODWELL	Biotic impoverishment and the threshold dilemma: A major challenge for science and government

NEUROBIOLOGY

(Offered jointly by Boston University Marine Program and the Marine Biological Laboratory)

I. INSTRUCTORS

ALAN FEIN, Marine Biological Laboratory, director of course
 FREDERICK LANG, Boston University Marine Program, director of course
 EDWARD F. MACNICHOL, JR., Marine Biological Laboratory, laboratory director

II. SPECIAL LECTURERS

WILLIAM J. ADELMAN, NIH-NINCDS, Woods Hole
 DANIEL L. ALKON, NIH-NINCDS, Woods Hole
 JELLE ATEMA, Boston University Marine Program
 ROBERT BARLOW, Syracuse University
 THOMAS CAREW, Columbia University
 MELVIN COHEN, Yale University
 VINCENT G. DETHIER, University of Massachusetts
 FREDERICK DODGE, Rockefeller University and IBM Watson Labs
 ERIC FRANK, Harvard Medical School
 DONALD FRAZIER, University of Kentucky
 ALFREDO GORIO, The Rockefeller University
 RAMI GROSSMAN, NIH-NINCDS
 ADRIANUS KALMIJN, Woods Hole Oceanographic Institution
 EHUD KAPLAN, Rockefeller University
 CHARLES LENT, State University of New York at Stony Brook
 SIMON LEVAY, Harvard Medical School
 DOUGLAS S. RIGGS, Hampshire College
 ELIZABETH S. RUSSELL, The Jackson Laboratory
 ARTHUR SILVERSTEIN, Johns Hopkins University

III. LECTURES

A. FEIN	Cell permeability and the plasma membrane
S. LEVAY	Microscopic anatomy of the nervous system
J. ATEMA	Evolution of chemical senses
M. COHEN	Evolution of neuron structure and function
A. KALMIJN	Physics and physiology of electroreception
A. FEIN	The resting membrane potential
A. FEIN	Cable properties of neurons
A. FEIN	Action potentials
A. FEIN	Saltatory conduction in myelinated axons
W. ADELMAN	Neuronal currents, channels, spaces and clefts
V. DETHIER	What chemosensory neurons tell the brain
R. BARLOW	The eye and the brain
R. BARLOW	Vision in <i>Limulus</i> : a multidisciplinary analysis
F. DODGE	A horseshoe crab-eye view of Great Harbor
E. KAPLAN	Receptor properties of <i>Limulus</i> lateral eye <i>in situ</i>
F. LANG	Synaptic transmission—The soups <i>vs.</i> the sparks
F. LANG	Mechanisms of transmitter release
F. LANG	Ionic basis of synaptic potentials
A. FEIN	Role of intracellular Ca^{++} and Na^{++} in adaptation of <i>Limulus</i> photoreceptors
F. LANG	Quantal release
F. LANG	Excitation-secretion coupling
E. RUSSELL	Pleiotropism and the nature of the W-locus in the mouse
F. LANG	Origin and fate of synaptic vesicles
F. LANG	Neurotrophic influences
A. GORIO	Intracellular recording of synaptic potentials
D. RIGGS	Pleasures and pitfalls of biological modeling
A. GORIO	Acetylcholine compartments in the mouse diaphragm
A. GORIO	The mode of action of black widow spider venom
L. MARSHALL	Factors influencing reinnervation of skeletal muscle
D. RIGGS	The steady-state behavior of biological feedback systems
E. FRANK	Formation of nerve-muscle synapses in tissue culture
D. RIGGS	Fitting straight lines when both X and Y are subject to error
F. LANG	Developmental neuroethology: Physiological basis for changes in fight and flight behavior during growth of the lobster
D. FRAZIER	Putative transmitters in vertebrate respiratory neurons
A. SILVERSTEIN	The generation of immunologic diversity: Phylogenists <i>vs.</i> ontogenists
E. KRAVITZ	Three neurohormones in the lobster: Studies on the cellular localization, release and physiological actions of octo- pamine, serotonin, and dopamine
C. LENT	Organizational and neural properties of leech CNS
R. GROSSMAN	Conduction of action potentials along nonhomogenous axons
T. CAREW	The utility of the marine mollusc <i>Aplysia</i> for the cellular analysis of behavior
C. LENT	Cellular studies of amine neurons in leech
D. ALKON	Associative training in a nudibranch mollusc, <i>Hermisenda</i>
D. ALKON	Neural substrates of associative training in <i>Hermisenda</i>

COMPARATIVE PATHOLOGY OF MARINE INVERTEBRATES

I. INSTRUCTORS

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 BETSY BANG, Johns Hopkins University
 JACK LEVIN, Johns Hopkins University
 ROBERT PRENDERGAST, Johns Hopkins University
 C. AUSTIN FARLEY, National Marine Fisheries Service at Oxford, Maryland
 KENNETH EDDS, Marine Biological Laboratory
 JACK MARCHALONIS, Frederick Cancer Research Center
 CAROL REINISCH, Sydney Farber Cancer Research Institute in Boston

II. LECTURES

F. B. BANG	Cellular clumping in seastars, hermit crabs, and clotting factors in <i>Carcinus</i>
J. LEVIN	Extracellular clotting
J. LEVIN	<i>Limulus</i> and endotoxin
J. LEVIN	Comparison of invertebrates and vertebrates
B. G. BANG	Evolution of mucociliary system
B. G. BANG	Sipunculus and urn cells
F. B. BANG	Comparative aspects of mucociliary systems
K. EDDS	Inflammations of Invertebrates
C. A. FARLEY	Normal Anatomy of the Oyster
C. A. FARLEY	Pathology of the Oyster
C. A. FARLEY	Infectious Diseases in the Oyster
C. A. FARLEY	Tumors in Oysters
C. A. FARLEY	Virus Diseases in Oysters
F. B. BANG	Specific Bacteriological and Virus Diseases of crabs and lobsters
J. PEARCE	Pathological pollution aspects of ecology
C. REINISCH	Tumors and regeneration
C. REINISCH	Recognition in invertebrates
R. PRENDERGAST	Starfish factor—effects in vertebrates
F. B. BANG	Elements of immune response—Sipunculus, stars
R. PRENDERGAST	Introduction to the vertebrate immune response
J. MARCHALONIS	Agglutinins and antibodies
J. MARCHALONIS	The evolution of the immune response

LABORATORY OF BIOPHYSICS, NINCDS-NIH

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 DANIEL L. ALKON, head, section on neural systems
 ALAN J. HODGE senior scientist; JAY WELLS, research physiologist; DAVID E. GOLDMAN, guest worker; TERRY CROW, staff fellow; IZIA LEDERHENDLER, ROBERT J. FRENCH, MITSUO TABATA, visiting fellows; JONATHAN SHOUKIMAS, THOMAS JERUSSI, LEON SHIMAN, IPA fellows; JOSEPH NEARY, biochemist; RICHARD WALTZ, mathematician/programmer; CLYDE TYNDALE, electronics specialist; RUTHANNE MUELLER, research technician; JUNE HARRIGAN, mariculturist

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 ALAN FEIN, associate scientist and deputy director; FERENC I. HÁROSI, associate

scientist; ETE Z. SZUTS, assistant scientist; BARBARA ANN COLLINS, senior research associate; D. WESLEY CORSON, postdoctoral fellow; JOSEPH LEVINE, graduate student, Harvard University; KATHLEEN FRENCH, graduate student, Boston University Marine Program; STEVEN L. GOODMAN, research assistant; MENACHEM HANANI, National Institutes of Health; THEODORE P. WILLIAMS, Florida State University; YVETTE KUNZ RAMSAY, University of Dublin; LEO E. LIPETZ, Ohio State University; PAUL WITKOVSKY, GUIDO HASSIN, State University of New York at Stony Brook, visiting scientists

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INDEPENDENT YEAR-ROUND PROGRAMS

RAYMOND STEPHENS, principal investigator

MELANIE PRATT; MARY E. PORTER

SHINYA INOUÉ, principal investigator

ANDREW EISEN; MARK B. FIENBERG

KENNETH EDDS, principal investigator

NORMAN R. JARVIS

ERIC BALL, principal investigator

D. EUGENE COPELAND, principal investigator

JUDITH GRASSLE, principal investigator

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1977-1978

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the Woods Hole Oceanographic Institution, the Boston University Marine Program, and in regularly scheduled seminars with staff of The Ecosystems Center and others at the MBL.

THE STAFF

GEORGE M. WOODWELL, director, The Ecosystems Center
 DANIEL B. BOTKIN, associate scientist
 FRANCIS P. BOWLES, assistant scientist
 JOHN E. HOBBIE, senior scientist
 JERRY M. MELILLO, assistant scientist
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(Including all whose services began or ended during the year)

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- SUGIMORI, MUTSU YUKI, Research Assistant Professor, New York University Medical Center
- SUMMERS, JESSE W., Member, Institute for Cancer Research
- SUMMERS, ROBERT G., Associate Professor of Anatomy, State University of New York at Buffalo
- SUSSWEIN, ABRAHAM J., Research Fellow, Albert Einstein College of Medicine, Yeshiva University
- SZENT-GYÖRGYI, ANDREW G., Chairman, Department of Biology, Brandeis University
- SZENTKIRALYI-SZENT-GYÖRGYI, EVA M., Research Associate, Brandeis University
- TABATA, MITSUO, Research Fellow, Laboratory of Biophysics
- TAMM, SIDNEY, Associate Scientist, University of Wisconsin
- TAMM, SIGNHELD, Research Associate, University of Wisconsin
- TASAKI, ICHII, Chief, Laboratory of Neurobiology, NIMH, National Institutes of Health
- TENEICK, ROBERT E., Associate Professor of Pharmacology, Northwestern University
- TERAKAWA, SUSUMU, Visiting Associate, National Institutes of Health
- TIFFERT, TERESA, Assistant Professor, Department of Physiology, University of Maryland, School of Medicine
- TRINKAUS, J. P., Professor of Biology, Yale University
- TROLL, WALTER, Professor of Environmental Medicine, New York University Medical Center
- TROXLER, ROBERT F., Associate Professor of Biochemistry, Boston University, School of Medicine
- TWEDELL, KENYON S., Professor, University of Notre Dame
- TYTELL, MICHAEL, Postdoctoral Fellow, Case Western Reserve University, School of Medicine
- VAN HOLDE, K. E., Professor, Oregon State University
- VAN RAALTE, CHARLENE, Instructor, Hampshire College

VINCENT, WALTER S., Professor of Cell and Molecular Biology, University of Delaware, School of Life and Health Sciences
 WAGNER, RICHARD W., Postdoctoral Fellow, University of Miami, School of Medicine
 WATERS ROBERT S., Postdoctoral Fellow, The Rockefeller University
 WAXMAN, STEPHEN G., Associate Professor of Neurology, Harvard Medical School
 WEBER, ANNMARIE, University of Pennsylvania, School of Medicine
 WEISSMANN, GERALD, Professor of Medicine, New York University Medical Center
 WHITTAKER, J. RICHARD, Associate Professor, The Wistar Institute
 WIERCINSKI, FLOYD J., Professor, Department of Biology, Northeastern Illinois University
 WILLIAMS, T. P., Professor of Biological Sciences, Florida State University
 WILSON, DARCY B., Professor of Pathology, University of Pennsylvania, School of Medicine
 WOLF, DON P., Associate Research Professor, University of Pennsylvania, School of Medicine
 WOOLEY, JOHN C., Research Fellow in Biochemistry, Harvard University
 WORGUL, Basil V., Research Associate, Department of Ophthalmology, Columbia University
 WU, CHAU H., Assistant Professor of Pharmacology, Northwestern University
 YEH, JAY Z., Assistant Professor, Northwestern University
 ZIGMAN, SEYMOUR, Professor of Ophthalmology and Biochemistry, University of Rochester, School of Medicine and Dentistry
 ZIRKIN, BARRY R., Associate Professor, Division of Reproductive Biology, The Johns Hopkins University

Lillie Fellow, 1978

LE DOUARIN, NICOLE, Centre National de la Recherche Scientifique, Institut D'Embryologie, France

Alexander Forbes Lecturer, 1978

KRNJEVIC, KRESIMIR, Department of Research in Anaesthesia, McIntyre Medical Sciences Building, Canada

Rand Fellow, 1978

MENZEL, RANDOLF, Institut fur Tierphysiologie, Freien Universitat, Berlin, West Germany

Grass Fellows, 1978

ASHCROFT, FRANCES M., Grass Foundation Fellow, Department of Zoology, Cambridge University, United Kingdom
 DAY, JOHN W., Albert Einstein College of Medicine
 EDWARDS, DONALD H., Postdoctoral Fellow, Stanford University
 FRAZIER, DONALD, Professor, University of Kentucky, School of Medicine
 GOLDBERG, MARK T., Grass Fellow, Memorial University of Newfoundland, Canada
 LUND, ALBERT E., Postdoctoral Fellow, Northwestern University Medical School
 MEYER, DAVID J., Grass Fellow, Health Sciences Center, State University of New York at Stony Brook
 NASS, MENASCHE M., Research Fellow in Biology, California Institute of Technology
 PELLMAR, TERRY, Research Fellow, National Naval Medical Center, Bethesda
 QUANDT, FREDERICK N., Postdoctoral Fellow, University of California, Los Angeles
 SATTERLIE, RICHARD A., Grass Fellow, University of California, Santa Barbara
 SCOTT, SHERYL A., Postdoctoral Fellow, Carnegie Institution of Washington
 SULLIVAN, ROBERT E., Postdoctoral Fellow, University of Hawaii at Manoa
 WATSON, WINSOR H., III, Research Associate, University of Massachusetts
 ZOTTOLI, STEVEN J., Research Scientist II, Research Institute on Alcoholism

Macy Scholars, 1978

ARMSTRONG, EARLENE, Assistant Professor, University of Maryland
 HILL, ANITA V., Assistant Professor of Biology, Grambling State University

HOGAN, JAMES C., JR., Department of Anatomy, Howard University College of Medicine
 HOWZE, GWENDOLYN B., Assistant Professor, Texas Southern University
 IVENS, M. SUE, Associate Professor, Natural Sciences, Dillard University
 PORTER, CHARLES W., Associate Professor of Biology, San Jose State University
 WALKER, DOROTHY G., Associate Professor, Howard University

Summer Research Scholarships, 1978

(Steps Toward Independence)

ARMETT-KIBEL, CHRISTINE, Assistant Professor of Biology, University of Massachusetts
 BOURNE, GEORGE B., Assistant Professor, The University of Calgary, Canada
 CHUNG, SU-YUN, Research Fellow in Chemistry, Harvard University
 FUJIWARA, KEIGI, Assistant Professor of Anatomy, Harvard Medical School
 HOFFMAN, RICHARD J., Assistant Professor of Biological Sciences, University of Pittsburgh
 HUFNAGEL, L., Assistant Professor of Microbiology, University of Rhode Island
 KASS-SIMON, M., Assistant Professor of Zoology, University of Rhode Island
 LANGFORD, GEORGE M., Assistant Professor of Anatomy, Howard University, College of Medicine
 LEWIS, LARRY M., Assistant Professor of Biology, Millersville State College
 O'MELIA, ANNE F., Assistant Professor of Biochemistry, Louisiana State University Medical Center
 RUDY, BERNARDO, Assistant Professor of Physiology, Eastern Pennsylvania Psychiatric Institute
 SALZBERG, BRIAN, Assistant Professor of Physiology, University of Pennsylvania
 TREISTMAN, STEVEN N., Assistant Professor of Biology, Bryn Mawr College
 YOUNG, LILY Y., Assistant Professor of Environmental Microbiology, Stanford University

Research Assistants, 1978

ANDERSON, DAVID J., The Rockefeller University
 ANSTROM, JOHN, State University of New York at Buffalo
 ANTONELLIS, BLENDA, University of Rochester School of Medicine
 ARVAN, PETER, Yale University
 AUGUSTINE, GEORGE J., JR., University of Maryland
 BARNES, EDWARD S., Columbia University
 BATRA, RANJAN, Institute for Sensory Research, Syracuse University
 BEHRMAN, AMY, Swarthmore College
 BENNETT, HOLLY V. L., The Albert Einstein College of Medicine
 BODICK, NEIL, Columbia University
 BOSLER, ROBERT B., Harvard University Medical School
 BOYLE, MARY B., Yale University
 BRANDON, CHRISTOPHER, Baylor College of Medicine
 BUSH, JAMES, Rutgers College
 BYERS, H. RANDOLPH, Harvard Medical School
 CALLE, PAUL, University of Pennsylvania
 CAPCO, DAVID GEORGE, University of Texas
 CHAN, YUE-WAH STEVEN, Baylor College of Medicine
 CHELSKY, DANIEL, University of Oregon
 CHENEY, CLARISSA M., University of Toronto
 COHEN, RICHARD, Hunter College
 COLUCCI, BARBARA ANN, Herbert H. Lehman College, The City University of New York
 CRAWFORD, WILLIAM, University of Hawaii
 CRITZ, ANNE McELROY, University of Pennsylvania
 CROOP, ROBERT, University of Pennsylvania
 DAHL, ROBERT F., Princeton University
 DANDEKAR, PRAMILA, State University of New York at Buffalo
 DAVIDSON, DOROTHY E., University of Delaware
 DRAKE, PETER F., Bryn Mawr College
 EAGLE, JANE S., University of Connecticut Health Center
 EIGNER, E. ANN, University of New Mexico School of Medicine
 FALKOW, STEPHEN, New York University, School of Medicine

FIELD, NANCY, Wesleyan University
FINE, ALAN, University of Pennsylvania
FINKEL, TOREN, University of Pennsylvania School of Medicine
FITT, WILLIAM K., University of California, Santa Barbara
FLEMING, JUDY, University of Colorado
FRIZZELL, KAREN L., University of Massachusetts
GALVIN, PATRIC N., University of Colorado at Denver
GILLY, WILLIAM F., University of Pennsylvania
GLICKSMAN, MARCIE, Brown University
GONCALVES, MARK A., University of Colorado
GORDON, DORIA, Oberlin College
GREEN, JEFFREY D., State University of New York at Buffalo
GREENWALT, DALE, Iowa State University
GREGA, DEBRA S., University of Kentucky
GRUPP, STEPHEN, University of Cincinnati
HARRIS, ANDREW L., The Albert Einstein College of Medicine
HARRIS, VICKI KAY, Emory University
HEINRICHS, STEFAN
HENRY, JONATHAN J., University of Hawaii
HERLANDS, LOUIS, Yale University
HERSCH, STEVEN M., Boston University
HO, SIMON MING HUNG, University of Toronto, Canada
HOLMES, DON J., Washington University Medical School
HOWARD, LOUISA, Dartmouth College
HURST, TERRY W., University of Texas, Medical Branch
HUSE, WILLIAM, Albert Einstein College of Medicine
HUTTNER, SUSANNE, University of California, Los Angeles
HYLANDER, BONNIE, State University of New York at Buffalo
IMHOF, RUTH, Biozentrum University of Basel, Switzerland
JAEGER, RICHARD R., Hunter College, The City University of New York
JANUS, TODD, J., Northwestern University
JASLOVE, STEWART, Duke University Medical Center
JIMENEZ, RAMON J., University of Guadalajara, Mexico
JUNBLATT, JAMES E., University of Basel, Switzerland
KATAYAMA, ROBERT N., University of Pennsylvania
KENDALL, DEBRA A., Northwestern University
KIEHART, DANIEL P., University of Pennsylvania
KIRK, MARK D., Rice University
KOUMJIAN, LAUREN, Mount Holyoke College
LAUFER, MARC R., University of Pennsylvania
LEIBOWITZ, DAVID H., Columbia University
LESHER, SARAH, Yale University
LEVINTHAL, ADAM
LEWIS, STEPHEN, University of Cincinnati
LITTLEFIELD, PETER, University of Maryland
LIVINGSTONE, M. S., Harvard Medical School
LUEBBERT, JO ANN, State University of New York at Stony Brook
LUTTINGER, KARL M., Columbia University
MARSDEN, J. ELLEN
MEDEL, THOMAS H., The Wistar Institute
MEYER, MICHAEL A., University of North Carolina at Chapel Hill
MIR, FRANCOISE, University of Basel, Switzerland
MONTANARO, GEORGE D., University of Iowa
MORELLO, ROBERT S., University of Rochester
MORGAN, CHARLES R., Emory University
MORGAN, GINA, Howard University
MORRIS, JAMES R., Case Western Reserve University
NARAHASHI, TARO, Northwestern University

NECLES, NICHOLAS, Columbia University
 NEMHAUSER, IRIS, Hunter College, The City University of New York
 NEUBIG, RICHARD R., Harvard Medical School
 PELLEY, CHIQUITA, University of Oregon
 PORTER, MARY E., University of Pennsylvania
 RAMOS, TALIA, The Johns Hopkins University School of Hygiene and Public Health
 REDMANN, GREG A., University of Texas Medical Branch
 REISS, PAUL, University of Maryland
 RENDER, JO ANN, University of Texas, Austin
 RHO, JAI-HYON, The Johns Hopkins University
 ROBERTSON, LOLA E., American Museum of Natural History
 ROLLINS, SHARON, Cornell University
 ROSENKRANZ, PNINA, Princeton University
 ROSMAN, GARY, New York University
 RUMER, NINA, University of Delaware
 SAMSON, DOUGLAS A., The Johns Hopkins School of Hygiene and Public Health
 SAUNDERS, MARY J., University of Massachusetts
 SCARBOROUGH, ANN, Louisiana State University
 SCHENCK, KATHLEEN, Princeton University
 SCHMIDT, JEFF, University of California, Los Angeles
 SCHMIDT, MADELYN R., Harvard Medical School
 SCHNEIDER, MARK ALAN, Amherst College
 SCRUGGS, VIRGINIA
 SHURE, MICHAEL S., Yale University
 SIEGEL, RUTH E., Harvard Medical School
 SIMON, SANFORD, New York University Medical Center
 SOCCI, ROBIN R., Rutgers, The State University of New Jersey
 SOLOMON, DENNIS J., Massachusetts Institute of Technology
 SOLWAY, ALAN, Wayne State University, School of Medicine
 STAMLER, JOHN F., University of Iowa
 STEELE, JOY ANN, University of Alberta, Canada
 STICH, THOMAS J., University of Maryland
 SUBYAK, SHARON E., Suffolk University
 SUH, KYUNGSUN, Columbia University
 SWAN, MICHAEL C., University of California, Los Angeles
 SWENSON, RANDOLPHE P., University of Pennsylvania
 TALIAN, JOHN, Carnegie-Mellon University
 TAYLOR, CHRISTOPHER E., The Johns Hopkins School of Hygiene and Public Health
 TIMPE, LESLIE C., Harvard Medical School
 TRAVIS, MARK A., Greenville College
 TYNER, EMILY M., Vassar College
 VON HIPPEL, DAVID F.
 WALDROP, BRIAN, Rice University
 WALTON, KERRY, New York University Medical Center
 WARREN, MARY K., University of Maryland
 WEEKS, JANIS C., University of California, San Diego
 WEISS, KEVIN, University of Pittsburgh
 WESTERFIELD, MONTE, Max Planck Institute fur Psychiatry, Germany
 WHEATLEY, RICHARD, Columbia University
 WICK, SUSAN M., University of Massachusetts
 WILLIAMS, CAROLYN H., Emory University
 WILLIAMS-ARNOLD, LOIS D., University of Hawaii
 WODLINGER, HAROLD M., University of Toronto, Canada
 YONEMOTO, WES, University of Hawaii
 YULO, TERESA, University of Rochester School of Medicine and Dentistry
 ZAKEVICIUS, JANE, New York University School of Medicine
 ZUKOWSKI, ANTHONY J., University of Hawaii

Library Readers 1978

- ADELBERG, EDWARD A., Professor of Human Genetics, Yale University
 ALLEN, NINA STROMGREN, Assistant Professor, Dartmouth College
 ALLEN, ROBERT DAY, Professor and Chairman, Dartmouth College
 ANDERSON, EVERETT, Professor of Anatomy and Associate Director, Laboratory of Human Reproduction and Reproductive Biology, Harvard University Medical School
 BEAN, CHARLES P., Biophysicist, General Electric Company
 BOURNE, DONALD, Marine Research Inc.
 CANDELAS, GRACIELA C., Professor, University of Puerto Rico
 CARRIERE, RITA, Assistant Professor, State University of New York, Downstate Medical Center
 CHILD, FRANK M., Professor of Biology, Trinity College
 CLIFFORD, SISTER ADELE, Professor of Biology, College of Mount St. Joseph on the Ohio
 COLE, KENNETH S., Research Biophysicist Emeritus, National Institutes of Health
 CONDOURIS, GEORGE A., Professor and Chairman, New Jersey Medical School
 COPELAND, DONALD EUGENE, Professor of Biology, Tulane University
 CORNWELL, ANNE CRISTAKE, Assistant Professor of Ophthalmology, Montefiore Hospital and Medical Center
 COUCH, ERNEST F., Associate Professor of Biology, Texas Christian University
 DAVIS, BERNARD D., Professor, Harvard Medical School
 DEHN, PAULA F., Graduate Assistant, University of Southern Florida
 DETTBARN, WOLF-D., Professor of Pharmacology, Vanderbilt University, School of Medicine
 DUDLEY, PATRICIA L., Professor of Biological Sciences, Barnard College, Columbia University
 DUNDAS, IAN, Associate Professor, University of Bergen, Norway
 EBERT, JAMES D., President, Carnegie Institute of Washington
 EDDS, LOUISE L., Associate Professor, Zoology and Microbiology, Ohio University
 ELDER, HOWARD A., Professor of Medicine, Albert Einstein College of Medicine
 EISEN, HERMAN N., Professor of Immunology, Massachusetts Institute of Technology
 ENGLANDER, SOL WALTER, University of Pennsylvania
 FISCHMAN, DONALD A., Professor and Chairman, State University of New York, Downstate Medical Center
 FISHER, SAUL H., Clinical Professor of Psychiatry, New York University, School of Medicine
 FRANZINI-ARMSTRONG, CLARA, Associate Professor of Anatomy, University of Pennsylvania
 GABRIEL, MORDECAI L., Professor of Biology, Brooklyn College, City University of New York
 GAGNE, GERARD D., Doctoral Candidate, University of Maine, Orono
 GALATZER-LEVY, ROBERT M., Lecturer, Associate Attending Psychiatrist, University of Chicago and Michael Reese Hospital
 GOLDMAN, ROBERT D., Professor of Biological Sciences, Carnegie-Mellon University
 GOLDSTEIN, MOISE H., JR., Professor, The Johns Hopkins University
 GOUDSMIT, ESTHER M., Associate Professor, Oakland University, Rochester
 GOULD, STEPHEN JAY, Professor of Geology, Harvard University
 GRANT, PHILIP, Professor of Biology, University of Oregon
 HAUBRICH, ROBERT, Professor of Biology, Denison University
 HENLEY, CATHERINE, National Eye Institute, National Institutes of Health
 HILLMAN, PETER, Associate Professor, The Hebrew University of Jerusalem, Israel
 HINSCH, GERTRUDE W., Associate Professor, University of Florida
 HOCHSTEIN, SHAUL, Lecturer, The Hebrew University of Jerusalem, Israel
 HUBERMAN, MICHAEL H., Intern—Internal Medicine and Pre-doctoral Fellow, New York University
 HUETTNER, ROBERT J., Assistant Professor, Columbia University, School of Dentistry
 HUNTER, R. DOUGLAS, Assistant Professor of Biological Sciences, Oakland University, Rochester
 ILAN, JOSEPH, Professor, Case Western Reserve University, School of Medicine
 INOUE, SADAYUKI, Assistant Professor, McGill University, Canada
 IRELAND, LEONARD, Associate, Bermuda Biological Station
 ISENBERG, IRVIN, Professor of Biophysics, Oregon State University
 ISSELBACHER, KURT J., Mallinckrodt Professor of Medicine, Chief of the Gastrointestinal Unit, Harvard Medical School and Massachusetts General Hospital
 ISSIDORIDES, MARIETTA R., Research Professor in the Department of Psychiatry, University of Athens, Medical School, Greece

- JAVITT, NORMAN B., Professor of Medicine, Head, Division of Gastroenterology, Cornell University Medical College
- JOHNSON, WILLIAM H., Professor of Biology, Rensselaer Polytechnic Institute
- KALTENBACH, JANE C., Professor of Biological Sciences, Mount Holyoke College
- KARUSH, FRED, Professor of Microbiology, University of Pennsylvania, School of Medicine
- KEAN, EDWARD L., Associate Professor, Case Western Reserve University
- KEOSIAN, JOHN P., Author
- KIRSCHENBAUM, DONALD M., Associate Professor, State University of New York, Downstate Medical Center
- KLEIN, MORTON, Professor of Microbiology, Temple University Medical School
- KOBAYASHI, MAKOTO, Professor of Physiology, Hiroshima University, Japan
- KOGUT, MARGOT, Lecturer, King's College, London, England
- KRNJEVIC, K., Professor and Director, Department of Anaesthesia Research, McGill University, Montreal, Canada
- LADERMAN, AIMLEE, Doctoral Candidate, State University of New York at Binghamton
- LAZAROW, JANE K., Senior Research Analyst, Minnesota Department of Health
- LE DOUARIN, NICOLE, Professeur, Directeur de l'Institut d'Embryologie du C. N. R. S. et du College de France, Centre National de la Recherche Scientifique
- LEFEVRE, MARIAN E., Associate Scientist, Medical Department, Brookhaven National Laboratory
- LEIGHTON, JOSEPH, Professor and Chairman, Department of Pathology, Medical College of Pennsylvania
- LEVITAN, HERBERT, Associate Professor, University of Maryland, College Park
- LIEBERMAN, MICHAEL W., Associate Professor of Pathology, Washington University, School of Medicine, St. Louis
- LINCK, RICHARD W., Assistant Professor, Harvard Medical School
- LINEAWEAVER, THOMAS H., III, Author
- LUX, HANS DEITER, Max Planck Institute of Psychiatry, Germany
- MARSLAND, DOUGLAS, Research Professor Emeritus, New York University
- MASLAND, RICHARD H., Assistant Professor of Physiology
- MITCHELL, JAMES B., Associate Professor of Biology, Moravian College
- MIZELL, MERLE, Professor of Biology, Tulane University
- MORRELL, FRANK, Professor of Neurology, Rush Medical College
- NEWBURY, THOMAS, Assistant Professor, University of Hawaii
- OJAKIAN, GEORGE, Assistant Professor, State University of New York, Downstate Medical Center
- PALMER, JOHN D., Chairman and Professor of the Zoology Department, University of Massachusetts, Amherst
- PEARLMEN, ALAN L., Associate Professor of Physiology and Neurology, Washington University, School of Medicine
- PLOCKE, DONALD J., Associate Professor and Chairman of Biology, Boston College
- ROSENBAUM, JOEL L., Professor of Biology, Yale University
- ROSENBERG, EVELYN, Professor, Jersey City State College
- SALMON, EDWARD D., Assistant Professor, Zoology Department, University of North Carolina, Chapel Hill
- SANGER, JOSEPH W., Associate Professor, University of Pennsylvania
- SAUNDERS, JOHN W., Professor, State University of New York at Albany
- SCHLESINGER, WALTHER R., Professor and Chairman, Department of Microbiology, Rutgers Medical School, College of Medicine and Dentistry of New Jersey
- SCOTT, GEORGE T., Professor of Biology, Oberlin College
- SHEMIN, DAVID, Professor and Chairman, Department of Biochemistry and Molecular Biology, Northwestern University
- SHEPRO, DAVID, Professor, Boston University
- SMITH, MICHAEL A., Research Associate, Hunter College, The City University of New York
- SONNENBLICK, B. P., Professor Emeritus, Rutgers, The State University of New Jersey
- SPECTOR, ABRAHAM, Professor of Ophthalmic Biochemistry, Department of Ophthalmology, Columbia University
- SUGDEN, BILL, Assistant Professor, University of Wisconsin
- SZAMIER, R. BRUCE, Assistant Professor, Harvard Medical School
- TRAGER, WILLIAM, Professor, The Rockefeller University

WAINIO, WALTER, Professor and Chairman, Department of Biochemistry, Rutgers College, Rutgers, The State University of New Jersey
 WAKSMAN, BYRON H., Professor of Pathology, Yale University
 WEBB, H. MARGUERITE, Professor of Biological Sciences, Goucher College
 WEISS, LEON, Chairman, Department of Animal Biology; Professor of Cell Biology, University of Pennsylvania, School of Veterinary Medicine
 WHEELER, GEORGE E., Professor of Biology, Brooklyn College, Biology Department
 WILSON, THOMAS H., Professor of Physiology, Harvard Medical School
 WITTENBERG, JONATHAN B., Professor of Physiology, Albert Einstein College of Medicine
 WOLF, JASON, Associate Professor, Wesleyan University
 YNTEMA, C. L., Professor Emeritus, Upstate Medical Center, State University of New York
 ZACKS, SUMNER I., Professor and Chairman, Section of Pathology, Brown University; Pathologist-in-Chief, Laboratory Director, The Miriam Hospital

Students, 1978

All students listed completed the formal course programs. Asterisk indicates those completing post-course research programs.

Summer Programs 1978

MARINE ECOLOGY

*BAXTER, MOLLY	KATZ, LAWRENCE C.
BRONSON, BECKY	*KIRCHMAN, DAVID LOUIS
CHEN, CELIA Y.	LECHLEITNER, RICHARD A.
*COPLY, NANCY JEANETTE	*MOGAN, ARTHUR
*DAY, MARGARET ELISABETH	*RIETSMAN, CAROL S.
DEMUTH, ROBERT EMERSON	ROSLANSKY, LOUISE
DONN, THEODORE E., JR.	SILBERHORN, ERIC MARTIN
*FAGAN, JOANNE	SKINNER, CAROL ANN
FREDERICQ, SUZANNE	*TALBERT, JEAN E.
*JENSEN, CYNTHIA LUND	

EMBRYOLOGY

ANDERSON, KATHRYN VIRGINIA	MUNEOKA, KEN
BROWN, KENNETH MICHAEL	RICE, DOUGLAS
CHILTON, BEVERLY SUE	SALIK, JANE ANNE
EDWARDS, MARY KAYE	SANDRIDGE, PAUL TIMOTHY
FLASTER, MURRAY S.	SCHULZ, STEPHANIE
GIBSON, BARBARA LOUISE	SZARO, BEN GREGORY
GUIDICE, GEORGE J., JR.	TANSEY, TERESE R.
JOHNSON, THOMAS EUGENE	WANER, NANCY LYNN
KORNHER, JOHN STEPHEN	WATANABE, MICHIO
KOSOFKY, BARRY EVAN	WEHRMAKER, ALFRED
LANDZBERG, JOEL SERGE	WISE, LAWRENCE DAVID
MARUSICH, MICHAEL F.	YOUNG, NEVIN DALE YOUNG
MILLER, CAROL LYNN	

NEURAL SYSTEMS AND BEHAVIOR

*BLEISCH, WILLIAM VIRGIL	HURLBERT, ANYA
CONDON, TIMOTHY	HURWITZ, JODIE LINDA
COQUELIN, ARTHUR	JAVITT, DANIEL C.
DAVID, WILLIAM S.	KUWADA, JOHN
EPEL, DEBRA LYNN	*LEVY, DANIEL JONATHAN
FURBISH, DEAN RUSSELL	MARCOTTE, RONALD R.
HOLZ, GEORGE G., III	MCMANUS, MARY ELLEN

MEHLER, BRUCE LEO
 *MILLER, MARK W.
 MOISEFF, ANDREW
 OLSON, LEAH M.
 ORR, DOMINIC PING-YIM
 *READY, NEAL EDWARD
 ROSE, BARRY
 *ROSE, ROBERT D.
 ROSELLI, CHARLES EUGENE

SCHESSEL, DAVID
 SCHILDBERGER, KLAUS
 *SCHMITT, BRIAN C.
 *SOLON, MICHAEL H.
 TANELIAN, DARRELL LEE
 TOMPKINS, LAURIE
 *TREVARROW, WILLIAM W.
 TURNER, JEFFREY SCOTT
 WEI, AGUAN DANIEL

EXPERIMENTAL MARINE BOTANY

ASHENDORF, DEBRA S.
 BERENBERG, CATHY JO
 BOCZAR, BARBARA A.
 *DOLYAK, BARBARA LORRAINE
 DUNLAP, JULIE J.
 FAVREAU, MITCHELL R.
 FITTER, MINDY SUE

*GRAVES, JOSEPH L., JR.
 JOHNSON, PATRICIA A.
 KAUFMAN, LON
 MONAR, KENNETH
 PEARCE, ROBERT ELLIOTT
 SHELIN, JONATHAN LEE
 WHITTAKER, CARLA J.

NEUROBIOLOGY

DODD, JANE
 FUKADA, KEIKO
 GARCIA-ARRARAS, JOSE E.
 LEMOS, JOSE RAMON
 MARCEY, DAVID JAMES
 PINE, JEROME

ROOF, DOROTHY J.
 SCHNEIDERMAN, ANNE M.
 SEJNOWSKI, TERRENCE J.
 STOCKBRIDGE, NORMAN LANDER
 THOMAS, WILLIAM ERIC
 YODLOWSKI, MARILYN L.

PHYSIOLOGY

BARR, ALAN HOWARD
 *BECKWITH, KIRK D.
 BEHER, MICHAEL GARY
 CARMAN, ALICE BLANCHE
 CHANG, JANE
 COLLINS, ELIZABETH T.
 *COLLINS, MARY
 COOPER, JOHN A.
 *CUTLER, MIRIAM RUTH
 *DAYTON, ANDREW IMBRIE
 *GERSHON, NAHUM DAVID
 *GRIFO, JAMES ANTHONY
 HINTZE, T.
 JAFFE, LAURINDA ANN
 LIEBER, MICHAEL R.
 MARQUEZ-STERLING, NUMA R.
 MATTSON, JOAN CRAWFORD
 *MC CARTHY, ROBERT ALAN

*MULHOLLAND, JOYCE I.
 *OTTER, TIMOTHY
 *PICKETT, CECIL
 *PINE, RICHARD IRA
 *PROFFITT, JOHN HOUSTON
 *RAUSCH, STEVEN
 *REBERS, JOHN ERIC
 *ROBINSON, MARGARET S.
 *SELICK, HAROLD E.
 *SHAHIN, ROBERTA D.
 SULLIVAN, CHARLES HENRY, II
 *SULLIVAN, SUSAN JEAN
 *SUPRENANT, KATHY ANN
 *TRAVIS, JEFFREY L.
 *WOOD, STEVEN CHENAULT
 *WU, CHAO-TING
 *WYBAN, JAMES ALLEN

MICROBIAL ECOLOGY

*AMY, PENNY S.
 *BELLAS, CHRISTINE MARIE
 *GOLDFARB, DAVID SCOTT
 GRAHAM, JULIA BELL
 *KASTER, ALLAN GERARD

PASTER, BRUCE JAY
 POTRIKUS, CATHERINE J.
 REED, WILLIAM MICHAEL
 SISCOE, PEGGY JEAN
 WARD, BESS B.

January Programs 1978

BEHAVIOR

ASHKENAS, LINDA R.
BENI, JANINE E.
BROWN, ADOLF MAXIMILIAN
BRYANT, BRUCE P.
CLARK, STEPHEN J.
DERBY, CHARLES DORSETT
DUWALDT, SARAH
ELGIN, RANDALL HULL
HERNANDEZ, TERESA

JONES, MAURICE, JR.
MILLER, DEBORAH LYNN
OBIN, MARTIN S.
POETHKE, DAVID JOHN
RAGLAND, HARRY CRAIG
STRATTON, ANDREA WOOD
WILLIAMS, ISABELLE P.
WOODBURY, PATRICK B.

DEVELOPMENTAL BIOLOGY

BARBACCI, JOSEPH J., JR.
BRODELL, GEORGE KLINE
BRYAR, BETSY A.
BURKE, DANIEL J.
EVARTS, JONATHAN HAROLD
FORD, WILLIAM C.
GILLESPIE, LAURA LEE
GOLD, ALBERT MARK
GUSTAFSON, KIRK ROBERT
HOLCOMB, CHERIE
HOWELL, MARGARET A.
MCGIMSEY, WILLIAM CLAYWELL

MCMANUS, GERALDINE A.
MANNING, MICHAEL SCOTT
MILANI, SUSAN
PATTERSON, NANCY
PERRY, GAIL E.
RHINEHART, MARY ELLEN
RIDDELL, DEBORAH CHRISTIE
SMILEY, LAURA ELLEN
TOTH, LESLIE ELLEN
TURNBULL, SARAH W.
VOWELL, JOANNA LYNN

ECOLOGY

ABOYA, ELLEN GWEN
BEHL, ANN
BERGANTZ, JAMES M.
BIXLER, ROBERT P.
COUPARD, MICHELE
DEPELTEAU, AUDREY MARIE
DETWILER, RALPH PAUL
FARRELL, THOMAS A.
FELDMAN, IRA ROBERT
GREENE, GEORGE D.
GROFF, JOSEPH MATTHEW
HOCHBERG, ANN PATRICIA
KELLEY, JULIA CROCKER

KELLOGG, DEAN LUNDT, JR.
LARSON, WENDELIN MAE
LAWRENCE, VIKI ANN
LEAMAN, DANNA JO
MACFARLANE, LINDA LOUISE
MACWHORTER, SUSAN E.
MCGOWAN, MICHAEL F.
NAUGHTON, DARLENE A.
PERFETTI, PATRICIA ANN
STEELE, PAMELA CURTIS
THEBY, JANET ELAINE
WALKER, SHARON LESLIE

NEUROBIOLOGY

AABY, TRYGVE GENE
APATAOFF, BRIAN R.
BAGGOTT, BRIAN BURKE
BODENSTEIN, LAWRENCE E.
CARR, CATHERINE EMILY
CHU, KA HOU
DILLSAVER, MARGARET
DOLBER, PAUL CHRISTIAN
GARBER, NANCY D.
HABER, MICHELE A.
HEINEKE, ERIC WAYNE
HIGGINS, GERALD ARTHUR
JAMES, DEMETRIA ELIZABETH
KENT, KARLA S.

KRASNOW, MARK ALAN
LOPEZ, JOSE R.
MACHLIS, LEE ELLEN
MORNAY, SHARON MARIE
MOULTON, JOHN FREEMAN, III
OGONOWSKI, MICHAEL M.
STEINBERG, ALAN B.
VALENTICH, JOHN D.
VERRETT, JOYCE M.
WEINBERG, BRADLEY ADAM
RIND, JEFF
SLADOVICH, HEDY E.
FEIGENBAUM, DAVID L.

COMPARATIVE PATHOLOGY OF MARINE INVERTEBRATES

BURNS, CARY DEWITT
 CARDENOSA, GILDA
 CARLSON, GEORGE A.
 CHORNEY, MICHAEL J.
 COOPER, KEITH RAYMOND
 COUCH, DAVID ROBERT
 DEAKINS, LYNN WHEELER
 GOLDBERG, HARRY R.
 GULKA, GARY J.
 HEATFIELD, BARRY MARK
 KELLER, THOMAS EARL

KEYT, BRUCE ALAN
 LOGUE, MAUREEN DENISE
 LOMBARDI, ROSEMARIE
 MILLER, TIMOTHY K.
 MULVEY, MARGARET E.
 NEWMAN, MICHAEL C.
 SEIFERT, RONALD A.
 SONSTEGARD, RONALD A.
 SYPEK, JOSEPH P.
 WALLACE, JAMES L.
 YEATON, ROBIN L. W.

BOSTON UNIVERSITY MARINE PROGRAM

AARONSON REBECCA
 ASHKENAS, LINDA
 BESSE, SHEILA
 BOTERO, LEONOR
 BRYANT, BRUCE
 BUCHSBAUM, ROBERT
 COLE, JAMES
 COLE, TIMOTHY
 CUSHMAN, MARY
 DAVIS, CABELL
 DERBY, CHARLES
 DOJIRI, MASAHIRO
 DOURDEVILLE, THEODORE
 DUNCAN, THOMAS
 EDER, SUSAN
 EISEN, JUDITH
 FABRIZIO, MARY
 FOREMAN, KENNETH
 FRENCH, KATHLEEN
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GIBSON, DANIEL
 HILL, RUSSELL
 HOWES, BRIAN
 JORDAN, THOMAS
 KENT, KARLA
 KIPP KATRINA
 KOLBA, CLIFFORD
 KOUMJIAN, LAUREN
 LANGBAUER, WILLIAM
 MACIOLEK, NANCY
 Ogonowski, MARK
 PASCOE, NATALIE
 PIOTROWSKI, MICHAEL
 POOLE, ALAN
 REID, ROBERT
 WALTHALL, WALTER
 WERME, CHRISTINE
 WIER, SUSAN
 WILLIAMS, ISABELLE
 WILSON, JOHN

YEAR-IN-SCIENCE

Spring 1978

WILLIAM B. BOWDEN
 SARAH M. MILLS
 JAMES T. MORRIS
 JAMES P. REED
 MARK N. WHITE

Summer 1978

WILLIAM B. BOWDEN
 AMY FRIEDLANDER
 RICHARD GRANT
 JAMES T. MORRIS
 JAMES P. REED
 MARK N. WHITE

Winter 1978

WILLIAM B. BOWDEN
 MICHAEL S. MANNING
 CHARLES P. McCLAGHERTY
 JAMES T. MORRIS
 JAMES L. OLDS
 JAMES P. REED

3. SCHOLARSHIPS, 1978

Bio Club:

Gary H. Calkins:

Lucretia Crocker:

Arthur Klorfein:

MARY ELLEN McMANUS

DOMINIC ORR
 ROBERT ROSE

BARBARA BO CZAR
 DAVID KIRCHMAN

WILLIAM BLEISCH
 DANIEL LEVY

TOSHIO NAGANO
NEAL READY
ROBERT ROSE
CHRISTOPHER TAYLOR

Josiah Macy, Jr.:

January

Summer

SHARON MORNAY
JOYCE VERRETT
WILLIAM FORD
MARGARET HOWELL

CECIL PICKETT
PATRICIA CHASE
CAROL BAGNELL
PATRICIA JOHNSON
WILLIAM THOMAS
JOSEPH GRAVES, JR.
PAUL SANDRIDGE
CARLA WHITTAKER

Society of General
Physiologists:

JOHN COOPER
MIRIAM CUTLER
ANDREW DAYTON

4. TABULAR VIEW OF ATTENDANCE, 1974-1978

	1974	1975	1976	1977	1978
INVESTIGATORS—TOTAL.....	508	511	535	501	522
Independent.....	302	301	312	280	268
Library Readers.....	75	81	93	82	106
Research Assistants.....	131	129	130	139	148
STUDENTS—TOTAL.....	158	212	249	212	250
<i>Summer Courses</i>					
Ecology.....	14	18	18		
Embryology.....	21	24	24	25	25
Experimental Invertebrate Zoology.....	30	34	36	33	
Experimental Marine Botany.....	11	14	16	16	14
Marine Ecology.....				19	22
Neural Systems and Behavior.....					31
Neurobiology.....	12	12	12	12	12
Physiology.....	40	33	41	34	34
<i>January Courses</i>					
Behavior.....		17	17	20	17
Biosphere.....		17			
Comparative Pathology of Marine Invertebrates.....					22
Developmental Biology.....	30	20	32	14	21
Ecology.....			29	17	25
Neurobiology.....		23	24	22	27
TRAINEES—TOTAL.....	41	31	9	10	11
TOTAL ATTENDANCE.....	707	754	793	723	783
Less persons represented in two categories.....	0	0	1	0	0
	707	754	792	723	783
INSTITUTIONS REPRESENTED—TOTAL.....	222	237	234	226	196
FOREIGN INSTITUTIONS REPRESENTED.....	31	26	33	33	30

5. INSTITUTIONS REPRESENTED, 1978

Albert Einstein College of Medicine
American Museum of Natural History
Amherst College
Baylor College

Boston College
Boston University
Boston University School of Medicine
Brandeis University

- Brookhaven National Laboratory
 Brooklyn College, The City University of New York
 Brown University
 Bryn Mawr College
 California Institute of Technology
 California, University of, Davis
 California, University of, Irvine
 California, University of, Los Angeles
 California, University of, San Diego
 California, University of, Santa Barbara
 Carnegie Institution of Washington
 Carnegie-Mellon University
 Case Western Reserve University
 Case Western Reserve University, Medical School
 Chicago, University of
 Cincinnati, University of
 College of Mount St. Joseph on the Ohio
 Colorado, University of
 Columbia University
 Columbia University, College of Physicians and Surgeons
 Columbia University, School of Dentistry
 Connecticut, University of
 Connecticut, University of, Health Center
 Cornell University
 Cornell University Medical College
 Dartmouth College
 Delaware, University of
 Denison University
 Dillard University
 Duke University
 Duke University Medical Center
 Eastern Pennsylvania Psychiatric Institute
 Emory University
 Florida, University of
 Florida State University
 General Electric Company
 Goucher College
 Grambling State University
 Grass Foundation
 Greenville College
 Hahnemann Medical College
 Hampshire College
 Hartford, University of
 Harvard Medical School
 Harvard University
 Hawaii, University of
 Herbert Lehman College, The City University of New York
 Howard University
 Howard University, College of Medicine
 Hunter College, The City University of New York
 Illinois, University of
 Illinois, University of, College of Medicine
 Illinois Institute of Technology
 Institute for Basic Research in Mental Retardation
 Institute for Cancer Research, The Iowa, University of
 Iowa State University
 Jersey City State College
 John B. Pierce Foundation Laboratory
 Johns Hopkins University, The
 Johns Hopkins University, The School of Hygiene
 Johns Hopkins University, The School of Medicine
 Kansas, University of
 Kent State University
 Kentucky, University of
 Kresge Eye Institute
 Laboratory of Biophysics, NINCDS-NIH
 Louisiana State University
 Louisiana State University Medical Center
 Maine, University of, Orono
 Manchester Community College
 Maryland, University of
 Maryland, University of, School of Medicine
 Massachusetts, University of
 Massachusetts General Hospital
 Massachusetts Institute of Technology
 Miami, University of
 Miami, University of, School of Medicine
 Millersville State College
 Minnesota Department of Health
 Minnesota, University of
 Miriam Hospital, The
 Mount Holyoke College
 Montefiore Hospital & Medical Center
 Moravian College
 National Institute of Mental Health, NIH
 National Naval Medical Center
 New Jersey Medical School
 New Mexico, University of, School of Medicine
 New York University
 New York University Medical Center
 New York University School of Medicine
 North Carolina, University of, at Chapel Hill
 North Carolina State University, Raleigh
 Northeastern Illinois University
 Northwestern University
 Notre Dame, University of
 Oakland University
 Oberlin College
 Ohio State University
 Ohio University
 Oregon, University of
 Oregon State University
 Pennsylvania, University of, School of Medicine
 Pennsylvania, University of, School of Veterinary Medicine
 Pennsylvania State University
 Pittsburgh, University of
 Population Council, The

Princeton University	State University of New York at Stony Brook
Public Health Research Institute of The City of New York, Inc.	Suffolk University
Purdue University	Swarthmore College
Michael Reese Hospital	Syracuse University
Rensselaer Polytechnic Institute	Temple University Medical School
Research Institute on Alcoholism	Texas Christian University
Rhode Island, University of	Texas Southern University
Rice University	Texas, University of, Austin
Rochester, University of	Texas, University of, Medical Branch
Rochester, University of, School of Medicine & Dentistry	Trinity College
Rockefeller University, The	Tulane University
Rush Medical College	Union Carbide Corporation
Rutgers—The State University of New Jersey	Vanderbilt University
Rutgers University Medical School	Vassar College
San Jose State University	Veterans' Administration Hospital, Brooklyn
South Florida, University of	Virginia, University of
Stanford University	Virginia, University of, School of Medicine
Stanford University School of Medicine	Washington University School of Medicine
State University of New York, Downstate Medical Center	Wayne State University
State University of New York, Upstate Medical Center	Wesleyan University
State University of New York at Albany	Wisconsin, University of
State University of New York at Binghamton	Wistar Institute
State University of New York at Buffalo	Woods Hole Oceanographic Institution
	Yale University
	Yale University School of Medicine
	Yeshiva University

FOREIGN INSTITUTIONS REPRESENTED, 1978

Aarhus, University of, Denmark	Imperial College of Science & Technology, London, England, U.K.
Alberta, University of, Canada	Instituto Venezolano de Investigaciones, Venezuela
Athens, University of, Medical School, Greece	King's College, London, England, U.K.
Basel, The University of, Biocenter, Switzerland	Laval, Université, Canada
Bergen, University of, Norway	Max Planck Institute for Psychiatry, Munich, West Germany
Bermuda Biological Station	Memorial University of Newfoundland, Canada
Calgary, University of, Canada	National Cancer Center Research Institute, Japan
Cambridge, University of, England, U.K.	North Wales, University of, Wales, U.K.
Centre National de la Recherche Scientifique, France	Ottawa, University of, Canada
Centro de Biofisica Y Bioquimica, Caracas, Venezuela	Puerto Rico, University of
Chiba University, Japan	Toronto, University of, Canada
College de France, Paris, France	University College, London, England, U.K.
Free University, Berlin, Germany	University College, University of Dublin, Ireland
Guadalajara, University of, Mexico	Wales, University of, Bangor, U.K.
Hebrew University, The, of Jerusalem, Israel	
Hiroshima University, Japan	

6. FRIDAY EVENING LECTURES, 1978

June 30

J. RICHARD WHITTAKER.....Egg cytoplasmic determinants of tissue differentiation	
The Wistar Institute	

July 7

BEATRICE MINTZ.....Putting mutant genes into mice
Institute for Cancer Research
Ninetieth Anniversary Lecture

July 13

Thursday

KRESIMIR KRNJEVIC.....Intraneuronal factors that influence excitability
McGill University 1) Regulation of neuronal firing by internal Ca^{2+}
Alexander Forbes Lecturer
at the MBL

July 14

KRESIMIR KRNJEVIC.....2) Intracellular actions of synaptic transmitters

July 21

DANIEL MAZIA.....Mitotic centers
University of California, Berkeley

July 28

ROBERT H. BURRIS.....Omnipresent N_2 —how organisms convert it to
University of Wisconsin NH_3

August 4

STEPHEN JAY GOULD.....Ontogeny and phylogeny
Harvard University

August 11

HARRIS RIPPS.....A ray of light on night blindness
New York University
Medical Center

August 18

ROBERT H. WHITTAKER.....Theory of species diversity
Cornell University

August 25

DANIEL ALKON.....A neural basis for associative learning
Laboratory of Biophysics,
NINCDS, at the MBL

7. MEMBERS OF THE CORPORATION, 1978

Including Action of 1978 Annual Meeting

Life Members

- ADOLPH, DR. EDWARD F., University of Rochester, School of Medicine and Dentistry, Rochester, New York 14627
- BEAM, DR. HAROLD W., Department of Zoology, State University of Iowa, Iowa City, Iowa 52240
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VI. REPORT OF THE LIBRARIAN

This year we received some relief in the crowded stack area. Dr. Gross approved the purchase of 300 additional metal shelves to be added to the top stack in the wing. This stack was not filled to capacity when originally built, so we were able to fit nine banks of shelving along each side and five banks at the back without crowding the Reserve Desks on the top floor. This new shelf space will hold approximately three years' growth in journal acquisitions. Before the end of that period we expect to have additional space in the Lillie Building made available to the Library.

Relief for Library salaries was also achieved this year. Specific MBL employee salaries were presented to the Members of the Board and Executive Com-

mittee. The result was increased salaries for many, somewhat comparable to those at the Woods Hole Oceanographic Institution.

A thorough inventory of the book section revealed a total of 25,000 volumes. When added to the journal collection, the complete volume count is now 180,000.

For this annual printed record, I would like to include a part of the preface of Dr. Stephen J. Gould's new book, "Ontogeny and Phylogeny," Harvard University Press, 1977:

"... and, above all, to an institution that has its own humanity and seems to me more an organism than a place—the Library of the Marine Biological Laboratory at Woods Hole. Where else would an idiosyncratic worker like me find a library open all the time, free from the rules and bureaucracy that stifle scholarship and "protect" books only by guarding them from use. It is an anomaly in a suspicious and anonymous age. May it survive as it is, despite all the improbabilities."

VII. REPORT OF THE TREASURER

I am pleased to report that the Marine Biological Laboratory made progress in 1978 in reducing its operating deficit. In 1978, the operating loss was \$35,454, a reduction from \$85,414 in 1977. Operating results excluded approximately \$282,000 of depreciation charges recorded in each year.

Total revenues in 1978 amounted to \$2,502,077, an increase of \$420,583 over the prior year. The principal sources of the increased revenues were higher tuition as the result of expanded programs and increased overhead recovery due to a 5% increase in the overhead rate, coupled with increased year-round occupancy. In addition, unrestricted gifts increased by almost \$100,000.

Total 1978 expenses amounted to \$2,537,531, an increase of \$370,623 over 1977. This increase was principally due to performing critical plant repairs which had been deferred in prior years for budgetary reasons, expansion of the Continuing Education programs begun in late 1977, the addition of two new course offerings, and a significant increase in the volume of the Chemical Room. Of this \$2,537,531, recurring operating expenses represented \$2,418,697, an increase of \$251,789, or 12%, over 1977. In our report last year, we mentioned that our 1978 budget anticipated that such expenses would increase only 5% from the 1977 level. This unanticipated and unfavorable variation in the expense budget is mainly attributable to the additional Continuing Education course offerings and greater volume in the Chemical Room—both of which generated additional offsetting revenues.

Investment income amounted to \$341,846 in 1978, an increase of 6% over 1977. Total investments at December 31, 1978 were valued at \$5,605,947 at market and \$5,739,745 at cost. The return, as a percentage of average market value, was 6.2%.

As was mentioned to you last year, methods of improving the rate of return on investments were to be examined during 1978. Accordingly, based on the recommendation of the Investment Committee, the Executive Committee, at its February 1979 meeting, appointed Standish, Ayer & Wood, Inc. of Boston to be the investment advisor for all of the Laboratory's investment portfolios.

We have budgeted in 1979 for a further reduction in the operating deficit and our objective for 1980 is to record a modest surplus.

The following is a statement of the auditors:

To the Trustees of Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheets of Marine Biological Laboratory as of December 31, 1978 and 1977, and the related statements of current funds revenues, expenditures, and other changes and changes in fund balances for the years then ended. Our examinations were made in accordance with generally accepted auditing standards and, accordingly, included confirmation from the custodians of securities owned at December 31, 1978 and 1977, and such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances.

As more fully described in Note B to the financial statements, the Laboratory excludes certain costs of buildings and equipment from the balance sheet. In our opinion, generally accepted accounting principles require that such costs be included as investment in plant in the financial statements.

In our opinion, except for the effects on the financial statements of the matter discussed in the preceding paragraph, the aforementioned financial statements present fairly the financial position of Marine Biological Laboratory at December 31, 1978 and 1977, and its current funds revenues, expenditures and other changes and the changes in fund balances for the years then ended, in conformity with generally accepted accounting principles applied on a consistent basis.

Our examinations were made primarily for the purpose of rendering an opinion on the basic financial statements (pages 94 to 99, inclusive), taken as a whole. The summary of investments (page 102) although not considered necessary for a fair presentation of the financial position at December 31, 1978 and 1977, current fund revenues, expenditures and other changes, and the changes in fund balances for the years then ended in accordance with generally accepted accounting principles, is presented primarily for purposes of supplementary analysis. This additional information has been subjected to the audit procedures applied in the examination of the basic financial statements and, in our opinion, is fairly stated in all material respects in relation to the basic financial statements taken as a whole.

Boston, Massachusetts
March 30, 1979

COOPERS & LYBRAND

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1978 and 1977

<i>Current Funds:</i>	<i>Assets</i>	<i>1978</i>	<i>1977</i>
Unrestricted:			
Cash, including deposits at interest.....		\$ 382,396	\$ 517,513
Accounts receivable, net of allowance for uncollectible accounts of \$4,500 in 1978 and \$12,955 in 1977.....		699,708	540,743
Other assets.....		15,367	15,996
Due to restricted current funds.....		(278,075)	(294,485)
Due from invested funds.....		103,248	105,133
Total unrestricted.....		<u>922,644</u>	<u>884,900</u>
Restricted:			
Cash.....		18,973	87,013
Investments, at cost; market value: 1978— \$1,297,551; 1977—\$1,118,917 (Note A, Schedule I).....		1,297,530	1,112,683
Due from unrestricted current fund.....		278,075	294,485
Due from invested funds.....		260,967	173,191
Total restricted.....		<u>1,855,545</u>	<u>1,667,372</u>
Total current funds.....		<u>\$ 2,778,189</u>	<u>\$ 2,552,272</u>
<i>Invested Funds:</i>			
Cash.....		6,982	102,031
Investments, at cost; market value: 1978— \$3,950,184; 1977—\$3,799,434 (Note A, Schedule I).....		4,084,003	3,822,798
Due to unrestricted current fund.....		(103,248)	(105,133)
Due to restricted current funds.....		(260,967)	(173,191)
Total invested funds.....		<u>\$ 3,726,770</u>	<u>\$ 3,646,505</u>
<i>Plant Fund:</i>			
Land, buildings and equipment at cost (Note B)....		12,421,929	12,374,587
Less accumulated depreciation.....		4,060,407	3,778,545
Total plant fund		<u>\$ 8,361,522</u>	<u>\$ 8,596,042</u>

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1978 and 1977

Liabilities and Fund Balances

1978

1977

Current Funds:

Unrestricted:

Accounts payable and accrued expenses.	\$ 316,657	\$ 195,418
Deferred income.	61,432	62,131
Fund balance.	544,555	627,351
Total unrestricted.	<u>922,644</u>	<u>884,900</u>

Restricted:

Fund balances:

Unexpended gifts and grants.	1,777,677	1,597,841
Unexpended income of endowment funds. . .	77,868	69,531
Total restricted.	<u>1,855,545</u>	<u>1,667,372</u>
Total current funds.	<u>\$ 2,778,189</u>	<u>\$ 2,552,272</u>

Invested Funds:

Endowment funds.	2,174,027	2,172,160
Quasi-endowment funds.	934,143	934,143
Retirement fund (Note C).	618,600	540,202
Total invested funds.	<u>\$ 3,726,770</u>	<u>\$ 3,646,505</u>

Plant Fund:

Invested in plant.	8,361,522	8,596,042
Total plant fund.	<u>\$ 8,361,522</u>	<u>\$ 8,596,042</u>

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

STATEMENTS OF CURRENT FUNDS REVENUES, EXPENDITURES, AND OTHER CHANGES
for the years ended December 31, 1978 and 1977

	<i>Unrestricted</i>		<i>Restricted</i>		<i>Total</i>	
	1978	1977	1978	1977	1978	1977
<i>Revenues:</i>						
Instruction:						
Tuition.....	\$ 236,391	\$ 164,495	\$ 43,100	\$ 35,200	\$ 279,491	\$ 199,695
Grants and contracts:						
Government.....	7,360	7,468	215,759	128,197	223,119	135,665
Private.....			70,446	33,258	70,446	33,258
Research:						
Laboratory rentals.....	503,576	424,631			503,576	424,631
Grants and contracts:						
Government.....	206,741	166,970	861,515	646,497	1,068,256	813,467
Private.....	90,550	84,917	390,692	457,896	481,242	542,813
Dormitory.....	320,020	285,285			320,020	285,285
Dining Hall.....	157,270	134,454			157,270	134,454
Library.....	135,028	126,652			135,028	126,652
Biological Bulletin.....	86,171	85,430			86,171	85,430
Support departments:						
Research Services.....	187,741	142,420			187,741	142,420
Marine resources.....	79,822	85,131			79,822	85,131
Investment income.....	159,703	158,361	41,417	39,359	201,120	197,720
Gifts.....	240,563	158,850	132,864	101,013	373,427	259,863
Other.....	91,141	56,430			91,141	56,430
Total revenues.....	2,502,077	2,081,494	1,755,793	1,441,420	4,257,870	3,522,914

	<i>Unrestricted</i>		<i>Restricted</i>		<i>Total</i>	
	1978	1977	1978	1977	1978	1977
<i>Operating expenditures:</i>						
Instruction.....	194,993	123,037	219,587	71,349	414,580	194,386
Research.....	3,813	1,386	1,264,631	1,104,393	1,268,444	1,105,779
Scholarships and stipends.....			216,958	216,736	216,958	216,736
Dormitory.....	135,769	140,358			135,769	140,358
Dining Hall.....	149,249	130,349			149,249	130,349
Library.....	235,401	208,149			235,401	208,149
Biological Bulletin.....	87,273	77,165			87,273	77,165
Support departments:						
Research services.....	354,485	305,847	25,400	20,415	379,885	326,262
Marine resources.....	218,794	216,278	34	255	218,828	216,533
Administration.....	514,204	462,479	4,263	4,048	518,467	466,527
Plant operation.....	643,550	501,860	2,163	2,181	645,713	504,041
Total expenditures.....	2,537,531	2,166,908	1,755,793	1,441,420	4,293,324	3,608,328
Excess of expenditures over revenues.....	(35,454)	(85,414)	—	—	(35,454)	(85,414)
<i>Transfers and additions:</i>						
Excess of restricted gifts, grants and investment income received over amounts expended.....			188,173	99,834	188,173	99,834
From plant fund—proceeds of sale of plant assets.....		3,000				3,000
To plant fund—donated unrestricted real estate.....	(47,342)				(47,342)	
From quasi-endowment fund.....		335,469				335,469
Net transfers and additions.....	(47,342)	338,469	188,173	99,834	140,831	438,303
Net increase (decrease) in fund balances.....	\$ (82,796)	\$ 253,055	\$ 188,173	\$ 99,834	\$ 105,377	\$ 352,889

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY
STATEMENTS OF CHANGES IN FUND BALANCES
for the years ended December 31, 1978 and 1977

	<i>Current Funds</i>		<i>Invested Funds</i>		<i>Plant Fund</i>
	<i>Unrestricted</i>	<i>Restricted</i>	<i>Endowment</i>	<i>Quasi-Endowment</i>	
1977					
Balances at December 31, 1976	\$ 374,296	\$ 1,567,538	\$2,147,067	\$1,260,509	\$8,848,333
<i>Increases:</i>					
Unrestricted current fund revenues.....	2,081,494				
Grants and gifts.....		1,622,821			29,000
Realized net gains on sale of investments.....		142,588	25,093	9,103	20,966
Investment income.....					3,000
Proceeds of sale of equipment.....				50,150	
Addition to pension fund.....		35,200			
Tuition.....					
<i>Decreases:</i>					
Instruction, research and general expenditures.....	(2,166,908)	(1,441,420)			
Indirect costs.....		(259,355)			
Payments to pensioners.....					(19,159)
Depreciation.....					(281,291)
<i>Transfers—additions (deductions):</i>					
Proceeds of sale of equipment.....	3,000				(3,000)
Transfer to current funds.....	335,469			(335,469)	
Balances at December 31, 1977.....	627,351	1,667,372	2,172,160	934,143	8,596,042

	<i>Current Funds</i>		<i>Invested Funds</i>			<i>Plant Fund</i>
	<i>Unrestricted</i>	<i>Restricted</i>	<i>Endowment</i>	<i>Quasi-Endowment</i>	<i>Retirement</i>	
<i>Increases:</i>						
1978						
Unrestricted current fund revenues.....	2,502,077					
Grants and gifts.....		2,055,387				
Realized net gains on sale of investments.....		150,130	1,867		32,013	
Investment income.....					65,573	
Addition to pension fund.....						
Tuition.....		43,100				
<i>Decreases:</i>						
Instruction, research and general expenditures.....	(2,537,531)	(1,755,793)				
Indirect costs.....		(304,651)				
Payments to pensioners.....					(19,188)	(281,862)
Depreciation.....						
<i>Transfers—additions (deductions):</i>						
Transfer to plant fund.....	(47,342)					47,342
Balances at December 31, 1978.....	\$ 544,555	\$ 1,855,545	\$2,174,027	\$ 934,143	\$618,600	\$8,361,522

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

NOTES TO FINANCIAL STATEMENTS

A. *Significant Accounting Policies:**Basis of Presentation—Fund Accounting*

In order to ensure observance of limitations and restrictions placed on the use of resources available to the Laboratory, the accounts of the Laboratory are maintained in accordance with the principles of "fund accounting." This is the procedure by which resources are classified into separate funds in accordance with activities or objectives specified. In the accompanying financial statements, funds that have similar characteristics have been combined.

Externally restricted funds may only be utilized in accordance with the purposes established by the source of such funds. However, the Laboratory retains full control over the utilization of unrestricted funds. Restricted gifts, grants, and other restricted resources are accounted for in the appropriate restricted funds. Restricted current funds are reported as revenue when expended for current operating purposes. Unrestricted revenue is reported as revenue in the unrestricted current fund when received.

Endowment funds are subject to restrictions requiring that the principal be invested and only the income utilized. Quasi-endowment funds have been established by the Laboratory for the same purposes as endowment funds, however, any portion of these funds may be expended.

Investments

Investments purchased by the Laboratory are carried at cost. Investments donated to the Laboratory are carried at fair market value at date received. For determination of gain or loss upon disposal, cost is determined based on the specific identification method.

Investment Income and Distribution

The Laboratory follows the accrual basis of accounting except that investment income is recorded on a cash basis. The difference between such basis and the accrual basis does not have a material effect on the determination of investment income earned on a year-to-year basis.

Investment income includes income from the investments of specific funds and from the pooled investment account. Income from the pooled investment account is distributed to the participating funds on the basis of the market value at the beginning of the quarter, adjusted for the cost of any additions or disposals during the quarter.

Indirect Cost Recovery

The Laboratory recovers indirect costs by charging occupants of laboratory space fees based on a negotiated fixed indirect cost rate for the period the space was occupied. When actual rates are subsequently determined, the difference is reflected in the next negotiated fixed rate.

B. Land, Buildings and Equipment:

Following is a summary of the plant fund assets:

<i>Classification</i>	<i>1978</i>	<i>1977</i>
Land.....	\$ 639,693	\$ 639,693
Buildings.....	10,190,430	10,143,088
Equipment.....	1,591,806	1,591,806
	12,421,929	12,374,587
Less accumulated depreciation.....	4,060,407	3,778,545
	\$ 8,361,522	\$ 8,596,042

The original cost of land, buildings and related initial furnishing equipment is capitalized when the assets are acquired. The cost of subsequent additions and purchases, repairs and remodeling is expensed when incurred. Equipment and remodeling expenditures amounted to approximately \$125,000 and \$110,000 in 1978 and 1977, respectively.

Depreciation is computed using the straight-line method over estimated useful lives of 40 years for buildings and 20 years for equipment.

C. Retirement Fund:

The Laboratory has a noncontributory pension plan for substantially all full-time employees which complies with the requirements of the Employee Retirement Income Security Act of 1974. The actuarially determined pension expenses charged to operations in 1978 and 1977 were \$76,374 and \$64,277, respectively. The Laboratory's policy is to fund pension costs accrued.

D. Pledges and Grants:

As of December 31, 1978 and 1977, the following amounts remain to be received from previous gifts and grants for specific research and instruction programs, and are expected to be received as follows:

	<i>1978</i>	<i>1977</i>
1978		\$1,697,486
1979	\$2,196,201	115,000
1980	28,000	
	\$2,224,201	\$1,812,486

SCHEDULE I
MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1978 and 1977

	Cost		Market		Investment Income	
	1978	1977	1978	1977	1978	1977
<i>Invested Funds:</i>						
U. S. Government securities.....	\$ 610,661	\$ 444,638	\$ 594,205	\$ 448,295	\$ 35,718	\$ 40,923
Corporate bonds.....	689,185	770,012	583,572	676,789	42,969	48,681
Common stocks.....	2,080,664	2,080,655	2,123,631	2,189,492	131,333	125,462
Commercial paper.....	—	—	—	—	—	10,990
Preferred stocks.....	83,032	83,032	54,548	61,072	3,654	4,468
Units in combined pension fund.....	602,912	426,912	576,679	406,237	33,114	9,394
Real estate.....	17,549	17,549	17,549 (1)	17,549 (1)	—	—
Total.....	\$4,084,003	\$3,822,798	\$3,950,184	\$3,799,434	246,788	239,918
Less custodian fees.....	—	—	—	—	14,867	11,835
					231,921	228,083
<i>Current Restricted Funds:</i>						
U. S. Government securities.....	1,297,530	1,112,683	1,297,551	1,118,917	90,082	86,748

Current Unrestricted Funds:

Earned on corporate savings account.....

19,843

7,084

Net investment income.....

341,846

321,915

Disposition of investment income:

Restricted for current use:

Research.....

102,538

95,789

Scholarships.....

25,715

25,289

Library.....

21,877

21,510

Retirement fund.....

150,130 (2)

142,588 (2)

Unrestricted—utilized in current operations..

32,013

20,966

159,703

158,361

341,846

321,915

(1) At cost.

(2) \$41,417 in 1978 and \$39,359 in 1977 was utilized in current restricted operations.

CYTOLOGY AND POLYSACCHARIDE CYTOCHEMISTRY OF THE GILL OF THE AMERICAN EEL, *ANGUILLA ROSTRATA*

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Much work has been published on the gills of teleosts. (Vickers, 1961; Munshi, 1964; Steen and Kruijsse, 1964; Hughes and Grimstone, 1965; Newstead, 1967; Hughes and Wright, 1970). Morgan and Tovell (1973) and Morgan (1974) described the structure and development of secondary lamellae in gills of trout. Work on eel gills has included cytological studies. (Ogawa, 1962; Yamada and Yokote, 1975), electron microscopic investigations of gill fine structure (Mizuhira, Amakawa, Yamashina, Shirai, and Utida, 1969), and studies of osmotic adaptation of eels to freshwater and seawater for the Japanese eel, *Anguilla japonica*. Keys and Willmer (1932) described chloride-secreting cells in the common eel, *Anguilla vulgaris*; Getman (1950) and Doyle and Epstein (1972) reported on osmotic effects and adaptive changes of chloride cells in the American eel, *Anguilla rostrata*.

The present study describes cytological details of gill filaments and secondary lamellae of freshwater-adapted early juvenile and adult American eels. Arrangement and morphology of epithelium, gill rays, mucous-secreting cells, pillar cells, and blood spaces within secondary lamellae are described and illustrated. Polysaccharide cytochemistry as revealed by periodic-acid-Schiff (PAS) staining as well as Alcian blue reactions at various pH values and with increasing concentrations of $MgCl_2$ of all cell types and connective tissue are described; results are compared with similar tissues in the *Anguilla* species, and in teleosts in general.

MATERIALS AND METHODS

Fresh water-adapted juvenile and adult eels were obtained from the Aquaculture Laboratories, Mercer Generating Station, Trenton, New Jersey. Animals were killed by decapitation and gills were excised and fixed in Davidson's solution at room temperature for 24 hr. Tissues were processed and embedded in paraffin (Humanson, 1972); sections were cut at 5 μm .

Stains used were hematoxylin and eosin, periodic-acid-Schiff (PAS) (McManus, 1948), Feulgen Picro-Amido-black (modified from Farley, 1969), Alcian blue 8GX (Gurr, London) at pH 0.5 (Lev and Spicer, 1964), Alcian blue pH 2.6, and Alcian blue pH 5.7 with post-treatment in ascending concentrations of $MgCl_2$ (Scott and Dorling, 1965; Mowry, 1970).

Slides were examined and photographed with the Zeiss Photomicroscope II using Kodak SO-410 Monochrome Photomicrography film with a Kodak #66 Wratten gelatin filter.

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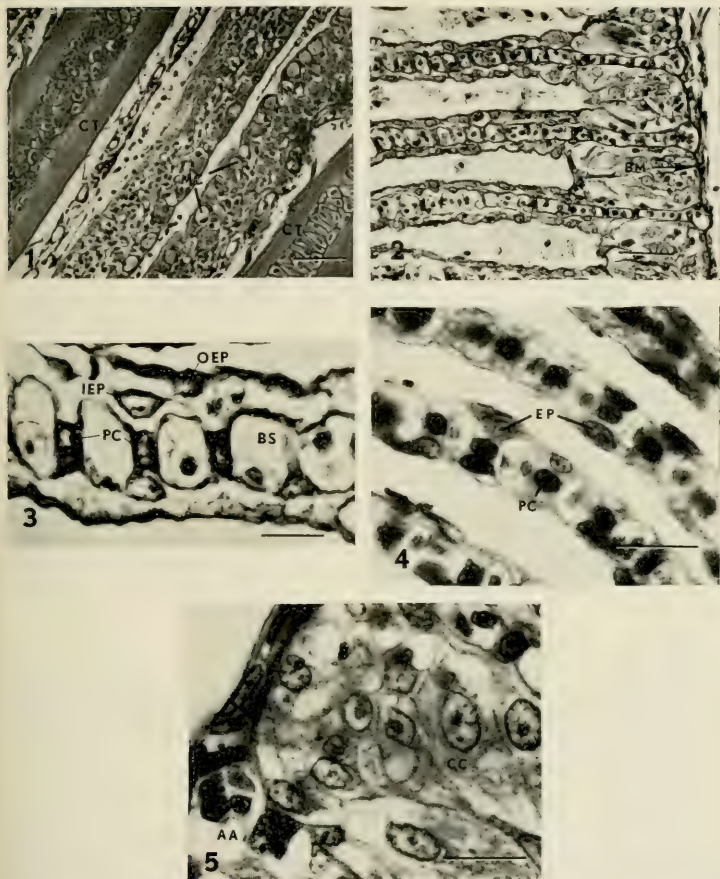


FIGURE 1. Cartilaginous gill rays (CT) support filaments of the eel gill. Mucus-secreting cells (MC) appear at bases of filaments. Hematoxylin and eosin stained; scale bar is 25 μ m.

FIGURE 2. Basement membrane (BM) in gill filament stained by PAS. Scale bar is 25 μ m.

FIGURE 3. Two-celled layered epithelium of secondary lamella of adult eel. Nuclei of inner cell layer (IEP) shown over pillar cell body (PC); pillar cells delimit blood spaces (BS) in lamellae. Note nucleus of outer layer of epithelium (OEP); scale bar is 10 μ m.

FIGURE 4. Secondary lamellae of early juvenile eel, showing single epithelial layer (EP) with nuclei over pillar cells (PC). Blood cells crowd blood spaces between pillar cells. Hematoxylin and eosin stained; scale bar is 10 μ m.

FIGURE 5. Chloride cells (CC) at the base of lamellae near afferent lamellar artery (AA). Note the prominent nucleolus, visible with Feulgen Picro-Amido-Black stain; scale bar is 10 μ m.

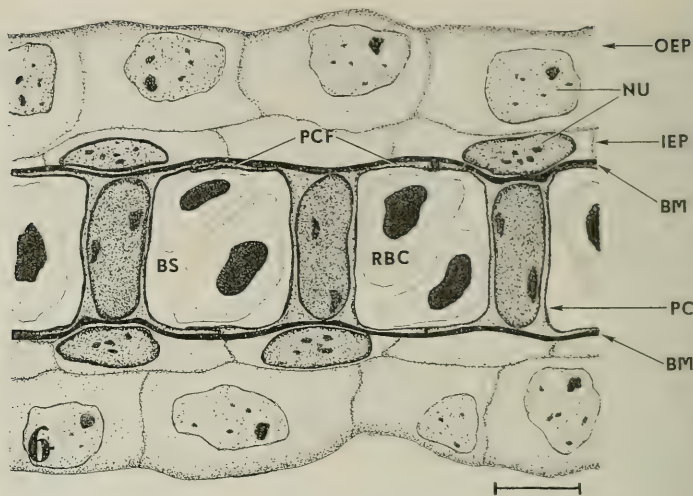


FIGURE 6. Diagram of secondary lamella of an adult eel. Pillar cells (PC) and their cytoplasmic flanges (PCF) delimit blood spaces (BS) within lamella. Two-cell layered epithelium supported by basement membrane (BM) covers secondary lamella. Nuclei (NU) of the inner epithelium (IEP) are more flattened than those of the outer epithelial cells (OEP) and lie over pillar-cell bodies. Scale bar is 5 μ m.

RESULTS

General morphology

Gills of the eel consist of four bony gill arches on either side of the pharynx. Gill arches bear two rows of flattened filaments supported for approximately two-thirds of their length by cartilagenous gill rays. Filaments each bear two rows of secondary lamellae.

Cytology and cytochemistry

Gill filaments contain a wide, centrally-located gill ray (Fig. 1) which stains intensely in PAS, Alcian blue pH 0.5, and Alcian blue pH 5.7 with high concentrations of MgCl_2 (Table I). Large mucus-secreting cells are found at both base and tip of filaments; cell secretions stain prominently with PAS reactions as well as with Alcian blue pH 2.6. They appear unstained in Alcian blue pH 0.5. A well-defined basement membrane supports the filament epithelium (Fig. 2); this connective tissue encircles afferent lamellar arteries and extends into secondary lamellae as the basement membrane for lamellar epithelia. It is stained intensely by PAS, Alcian blue pH 0.5, and Alcian blue pH 5.7 with concentrations of MgCl_2 up to 1.30 M (Table I).

Blood supplied by afferent lamellar arteries flows through blood spaces delimited by pillar cells and their cytoplasmic flanges (Figs. 3, 6). Pillar cell nuclei typically contain two small areas of chromatin material with fine granular material distributed around the margins of the nuclear envelope. Nuclei occupy nearly the entire volume of the cell, with a width of approximately $4\text{ }\mu\text{m}$ and a height of $8\text{ }\mu\text{m}$.

A two-layered epithelium covers secondary lamellae in adult eels, supported by a basement membrane continuous along pillar-cell flanges. Nuclei of inner epithelial layers frequently lie directly over pillar-cell bodies (Figs. 3, 6), with nuclei flattened against the basement membrane. Several dense areas of chromatin appear near the center of the nucleus, with smaller areas of chromatin adhering to the nuclear envelope. Nuclei of the outer epithelial layer appear more spherical than inner epithelial nuclei; one dark nucleolus is usually visible surrounded by smaller chromatin masses (Fig. 6). Cytoplasm of outer epithelial cells is much less dense than that of inner epithelial cells, with distal portions of cytoplasm staining lightly with Alcian blue pH 2.6. A single-layer epithelium covers secondary lamellae in early juvenile eels (Fig. 4); epithelial nuclei are located over pillar-cell bodies.

Chloride-secreting cells surround afferent lamellar arteries, crowded in the "v" between adjacent secondary lamellae (Fig. 5). Cells are large, with a granular, eosinophilic cytoplasm and a large nucleus with a prominent nucleolus surrounded by small regions of chromatin material (Fig. 5). Crowding of cells in this area of filaments causes them to be elongated, with nuclei typically located at the base of cells. Chloride-cell cytoplasm stains heavily in PAS reactions and exhibits some reaction to Alcian blue pH 2.6, especially at distal portions of cells. In Alcian blue pH 0.5 there is little reaction in chloride cells; further, in Alcian blue pH 5.7, cells reach their extinction point at a critical electrolyte concentration of 0.030 M MgCl_2 (Table I).

DISCUSSION

Cartilage supporting gill filaments shows heavy concentrations of sulfated mucopolysaccharides, staining with Alcian blue pH 0.5 (Lev and Spicer, 1964). Polysulfate groups stain selectively as pH is lowered to a point below the pH of carboxyl groups; sulfate groups still dissociated are free to bind the cationic dye. Mowry (1963) reported that, at low pH, hyaluronic acid, heparin, and chondroitin readily stain in Alcian blue; the color reactions are identical but chondroitin comprises only a minor portion of extracellular material. However, the sulfate ester derivatives, chondroitin sulfate A and chondroitin sulfate C, are major structural components of vertebrate cartilage (Lehninger, 1970). In Alcian blue pH 5.7 cartilage reacts intensely when treated with high concentrations of MgCl_2 (Table I). Mowry (1970) noted that binding of the cationic dye in 0.30 M or higher concentrations of MgCl_2 indicated the presence of sulfated polyanions; the higher the ionic strength of Mg^{2+} the higher the degree of sulfation. Addition of MgCl_2 dissolves the stained complexes formed by the polyanion's reaction with Alcian blue (Scott and Dorling, 1965). The critical electrolyte concentration (lowest concentration of Mg^{2+} at which a given polyanion is no longer stainable) is 1.20 M MgCl_2 for gill-ray cartilage. The presence of highly sulfated chondroitin

TABLE I
Results of cytochemical tests.

Stain	Mucous cells	Basement membrane	Cartilage	Chloride cells
Hematoxylin and eosin	+	++	+++	+++
PAS	+++++	++++	+++	+++
Feulgen-Picro Amido-black	++	++	+++	+++++
Alcian blue pH 0.5	---	+++++	++++	++
Alcian blue pH 2.6	++++	++	+++	++
Alcian blue pH 5.7 with				
0.00 M $MgCl_2$	+++	+++	+++	++
0.05 M $MgCl_2$	+++	+++	+++	++
0.10 M $MgCl_2$	+++	+++	+++	+
0.20 M $MgCl_2$	---	+++	+++	+
0.30 M $MgCl_2$	---	+++	+++	---
0.40 M $MgCl_2$	---	+++	+++	---
0.50 M $MgCl_2$	---	+++	+++	---
0.60 M $MgCl_2$	---	+++	+++	---
0.70 M $MgCl_2$	---	++++	+++	---
0.80 M $MgCl_2$	---	++++	+++	---
0.90 M $MgCl_2$	---	++++	++	---
1.00 M $MgCl_2$	---	++++	++	---
1.10 M $MgCl_2$	---	++++	++	---
1.20 M $MgCl_2$	---	+++++	++	---
1.30 M $MgCl_2$	---	+++++	---	---

---, No reaction; ++, weak reaction; +++++, strong reaction.

derivatives in cartilage accounts for the intensity of reactions to Alcian blue pH 0.5 and Alcian blue pH 5.7 with high concentrations of $MgCl_2$.

Mucous cells found on filaments at both base and tip have secretions which exhibit properties of acid mucopolysaccharides with vicinal hydroxyl as well as carboxyl groups. Cell secretions stain heavily in Alcian blue pH 2.6, with no staining in Alcian blue pH 0.5. Further, mucous cell secretions reach their extinction point at a critical electrolyte concentration of 0.20 M $MgCl_2$ in Alcian blue pH 5.7 series (Table I). Mowry (1963) stated that basophilia in a 0.30 M or lower concentrations of $MgCl_2$ indicated the presence of polycarboxylates. In addition, Alcian blue is a reliable and sensitive test for carbohydrate polycarboxylates.

Some variation in the exact chemical structure of mucus secretions apparently exists within the *Anguilla* species. Yamada and Yokote (1975) reported the presence of sulfated mucopolysaccharides in mucous cells of the Japanese eel, *Anguilla japonica*, and described staining of cells in a range from 0.10 to 0.60 M $MgCl_2$; those secretions show a higher degree of sulfation than those of *Anguilla rostrata* using similar procedures (Table I). Mucous cells always appear at the base and tip of gill filaments in the eel, as described for trout (Morgan and Tovell, 1973) and many other teleosts (Newstead, 1967; Hughes and Wright, 1970).

Electron microscope studies on epithelial basement membranes in gills from rainbow trout (Morgan and Tovell, 1973) and other teleosts (Hughes and Grim-

stone, 1965; Newstead, 1967) showed a close association of basement membrane and pillar-cell flanges. In the present study, epithelial basement membrane is also in contact with cytoplasmic flanges of pillar cells (Fig. 6); it also surrounds afferent lamellar arteries at bases of secondary lamellae (Fig. 2) and supports gill-filament epithelia. The basement membrane shows heavy concentrations of sulfated mucopolysaccharides, staining intensely in Alcian blue pH 0.5; in Alcian blue pH 5.7 the basement membrane can also be distinguished when treated with concentrations of $MgCl_2$ up to 1.30 M (Table I). Magnesium chloride provides better discrimination of polyanions than other salts (Scott and Dorling, 1965) and the critical electrolyte concentration is a reflection of the type of polyanions present, as well as the concentration. In *Anguilla rostrata*, basement membranes exhibit an even greater degree of sulfation than gill-ray cartilage (Table I).

Blood supplied to secondary lamellae is channeled through blood spaces formed by overlapping pillar cell flanges (Fig. 6). These blood spaces appear to be somewhat similar to capillaries; however, no endothelium could be discerned. Studies on trout (Morgan and Tovell, 1973) and other teleosts (Hughes and Grimstone, 1965; Newstead, 1967; Hughes and Wright, 1970) indicated that blood spaces in secondary lamellae were entirely delimited by extensions of pillar-cell cytoplasm. Morgan (1974) further stated that, developmentally, pillar cells originate directly from mesenchymal cells, and not from cells having affinities to endothelial cells.

Above the pillar cells and basement membrane a two-cell, layered epithelium covers secondary lamellae in adult eels (Fig. 3). Nuclei of inner epithelial cells lie directly over pillar cell bodies (Figs. 3, 6); similar arrangements have been reported to exist in trout (Morgan and Tovell, 1973) and many other teleosts (Newstead, 1967; Hughes and Wright, 1970). Hughes and Grimstone (1965) suggested that location of epithelial nuclei over pillar cell bodies could be adaptive, as little gas exchange would be expected at those points. In the present study, the average water-to-blood distance for adult eels is 5 to 8 μm , but at points where epithelial nuclei are located, the distance is nearly doubled from the free edge to the blood spaces. In early juvenile eels (2–3 g body weight) there is only one epithelial layer in secondary lamellae; nuclei of these cells invariably lie over pillar cell bodies (Fig. 4). Here, the water-to-blood distance is only 3 to 4 μm .

Keys and Willmer (1932) reported only a single layer of epithelium in secondary lamellae of the common eel, *Anguilla vulgaris*, but they gave no information concerning the size of animals used in their study. From the evidence of the two cell layers found in *Anguilla rostrata* and other teleosts, it is apparent that, as the animals mature, a second cell layer appears and the water-to-blood distance increases slightly.

Chloride-secreting cells appear in clusters at bases of secondary lamellae, in close proximity to afferent lamellar arteries (Fig. 5). Cytoplasm of chloride cells exhibits high concentrations of carbohydrate polycarboxylates staining intensely with PAS reactions (Table I) and Alcian blue pH 2.6. In the latter, it is interesting to note that staining is limited to the distal portions of chloride cells; basal portions of cells show no reaction to the stain.

Appearance of chloride cells near afferent lamellar arteries is well known for the Japanese eel, *Anguilla japonica* (Ogawa, 1962; Shirai and Utida, 1970; Utida,

Kamiya, and Shirai, 1971) as well as other teleosts (Vickers, 1961; Munshi, 1964; Newstead, 1967). Getman (1950) suggested that in *Anguilla rostrata*, the location of chloride cells in interlamellar epithelium allows access to a good blood supply and insures exposure to the environment for salt secretion. Shirai and Utida (1970) studied the development and degeneration of chloride cells when eels were adapted to fresh water and to sea water; the secretory mechanism was examined by Utida, *et al.* (1971) to determine the relationship between $\text{Na}^+\text{-K}^+\text{-ATPase}$ and numbers of chloride cells in seawater-adapted animals. In the present study, animals were freshwater adapted, and chloride cells were similar in appearance, location, and stainability to those of freshwater teleosts (Munshi, 1964).

In conclusion, we would like to thank Dr. Joseph A. Vena for his advice and assistance with staining procedures, and Mr. Gerald Nicholls for his suggestions and cooperation on photographic techniques.

SUMMARY

Gills of the American eel were found to be morphologically similar to those of other members of the *Anguilla* species, and to teleosts in general. Gill filaments contain cartilaginous gill rays rich in polysulfates, and stain intensely in PAS and Alcian blue pH 0.5.

Pillar cells delimit blood spaces within secondary lamellae; they were found to be covered by a thin connective tissue supporting a single-layered epithelium in early juvenile animals and a two cell, layered epithelium in adult eels. In the latter, nuclei of the outer layer were much larger and not as densely stained as those of the inner epithelial cells, whose nuclei appeared flattened over pillar-cell bodies.

Basement membranes supporting epithelia of secondary lamellae and gill filaments exhibited heavy concentrations of sulfate groups shown by reactions in Alcian blue pH 0.5 and Alcian blue pH 5.7 with high concentrations of MgCl_2 .

Chloride cells were found in the interlamellar epithelium, especially surrounding afferent lamellar arteries. They had a granular, eosinophilic cytoplasm with carbohydrate polycarboxylates concentrated in distal portions of cells; nuclei had a prominent, centrally-situated nucleolus surrounded by small chromatin masses.

Results of cytochemical tests for all cell types were reported, and information correlated to previous findings on eel gills in particular, and teleost gills in general.

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FINE STRUCTURE OF MUSCULATURE IN THE COPEPOD *PARANTHESSIUS ANEMONIAE* CLAUS

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Paranthessius anemoniae Claus is a cyclopoid associate of the snakelocks anemone *Anemonia sulcata* (Pennant). First described by Claus (1889) in the Adriatic Sea and later by Bocquet and Stock (1959) from Mediterranean waters, *Paranthessius* has only recently been recorded from British waters (Gotto and Briggs, 1972; Briggs and Gotto, 1973; Briggs, 1973). Other recent studies of *Paranthessius* have described this copepod's general ecology (Briggs, 1976), alimentary canal (Briggs, 1977a), larval development (Briggs, 1977b) and integument (Briggs, 1978). Copepod muscle has been investigated by Hartog (1888), Scott (1901), Lowe (1935), Changeux (1960), Fahrenback (1962) and Park (1966). Ultrastructural studies of *Cyclops* by Bouligand (1962, 1963 and 1964) and of *Macrocylops albidus* by Fahrenbach (1963) are among the few detailed studies of copepod muscle.

MATERIALS AND METHODS

Copepods were fixed for 12 hr at 4° C in 5% gluteraldehyde in 0.12 M Millonig buffer (pH 7.4) containing 3% NaCl and 0.1 mM CaCl. Fixed specimens were processed for light and electron microscopy.

Light microscopy

Copepods fixed in gluteraldehyde were dehydrated through ethylene glycol and embedded in glycol methacrylate (G.M.A.) which was polymerized in gelatin capsules at 60° C for 48 hrs. Sections 1 to 2 μ thick were cut on a Reichert OMU2 ultratome and stained on glass slides with mercuric bromo phenol blue (method of Maiza, Brewer, and Alfert, 1953).

Electron microscopy

Fixed copepods were washed in Millonig buffer-wash (2-8 hrs), post-fixed for 2 hrs in 1% osmium tetroxide and the dehydrated through ethanol to propylene oxide and embedded in araldite. Sections were cut with a Reichert OMU2 ultratome, mounted on copper grids, stained with uranyl acetate and lead citrate and examined in an AE1 EM801 electron microscope operating at 60 kV.

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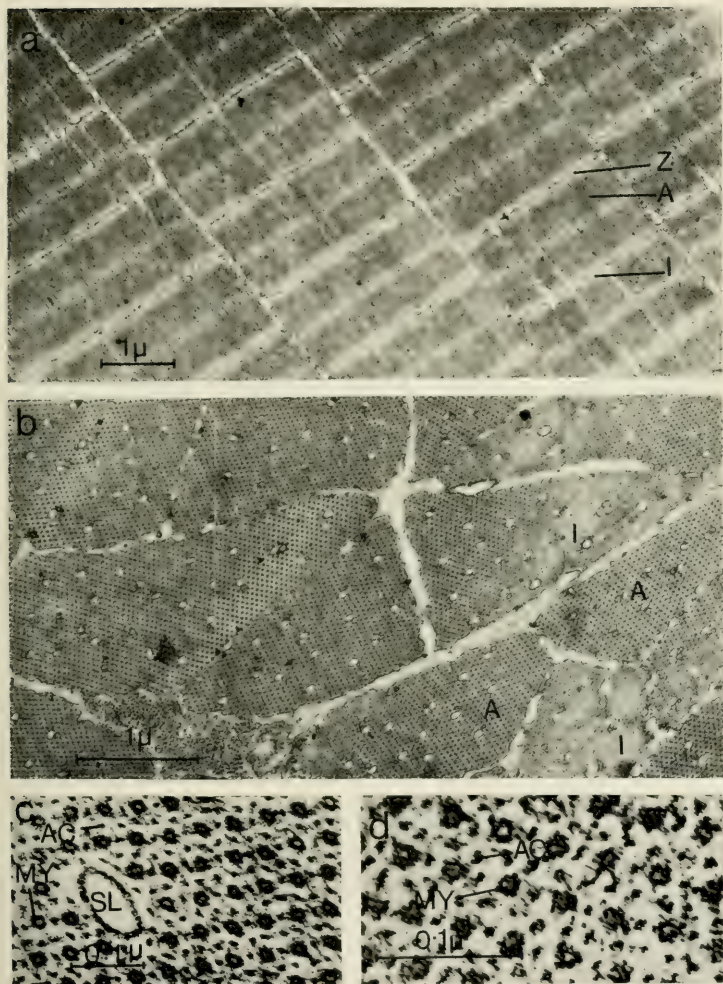


FIGURE 1. Electron micrograph of striated muscle in *Paranthessius anemoniae*. (a) Longitudinal section through muscle. Z line, A and I band are arrowed. (b) Transverse section of muscle fibrils at both A band and I band levels. (c) Transverse section of muscle showing longitudinal sarcoplasmic reticulum element (SL) actin filaments (AC) and myosin. (d) actin (AC) and myosin filaments (MY).

RESULTS

The general body muscle

Light microscopy has shown the longitudinal muscles in *Paranthessius* to be composed of bundles of muscle fibers which pass along either side of the mid-dorsal line. A similar pair of longitudinal muscle bundles are situated in a ventro-lateral position. Treatment with periodic-acid-Schiff (method of McManus, 1946) demonstrated the presence of large amounts of glycogen within the muscle.

Examination of ultra thin sections with the electron microscope shows the muscle of *Paranthessius* to be striated (Fig. 1a). A and I bands are clearly visible, the former having a well-defined central H zone as is usual for striated muscle (Hanson and Huxley, 1953). The functional units of muscle (sarcomeres) are separated from one another by an electron-dense Z line. Transverse section shows the muscle to be composed of polygonal-shaped myofibrils measuring between 1 and 4 μ in diameter (Fig. 1b). Both thick (myosin) and thin (actin) filaments are present. The myosin filaments, which appear to be hollow, measure 12 nm in thickness with an average length of 1100 nm and are spaced about 25 nm apart. The actin filaments, on the other hand, are on average 4 nm in thickness and are each placed equidistant between two myosin filaments. The sarcomere has an average length of 1200 nm, though this varies with the state of muscle contraction.

Superficial examination of *Paranthessius* muscle reveals an apparent similarity to vertebrate muscle with each myosin filament surrounded by six similar filaments and six actin filaments in hexagonal array (Figs. 1c, d). More detailed examination, however, shows that the actual position of the actin filaments in relation to the myosin filaments is different from vertebrate muscle in that each actin filament does not lie equidistant from three myosin filaments (Fig. 2). If the myosin filaments in *Paranthessius* are imagined to be the apices of an equilateral triangle, then the actin filaments occur in the center of each side (Fig. 2). This arrangement of myofilaments is similar to that described for other copepods, for example Bouligand (1962), Fahrenbach (1963) and Rayment, Krishnaswamy, Woodhouse, and Griffin (1974).

Sections through the muscle show the myofibrils to be surrounded by membranous material which constitutes the sarcoplasmic reticulum. This is also seen to be regularly distributed within the myofibrils. In transverse section, the interfibrillar sarcoplasmic reticulum appears as circular membranous zones, often paired, measuring 50 nm in diameter. The single or paired units are regularly spaced between 200 nm and 400 nm apart (Fig. 3a). Longitudinal sections show the sarcoplasmic reticulum to be in the form of canals measuring an average of 50 nm across and of varying length (Fig. 3b), and appearing elliptical in slightly oblique sections (Fig. 3c).

Examination of a large number of muscle sections has revealed that the sarcoplasmic reticulum is a branching tubular system that runs longitudinally through and around the myofibrils. The intrafibrillar elements link up with those surrounding the fibril in the region of the Z line by means of transverse tubules of sarcoplasmic reticulum. This feature is evident in both transverse and longitudinal sections (Fig. 3d, 4a). The canals surrounding the myofibrils are continuous with the membrane of the sarcolemma surrounding the muscles. This implies the

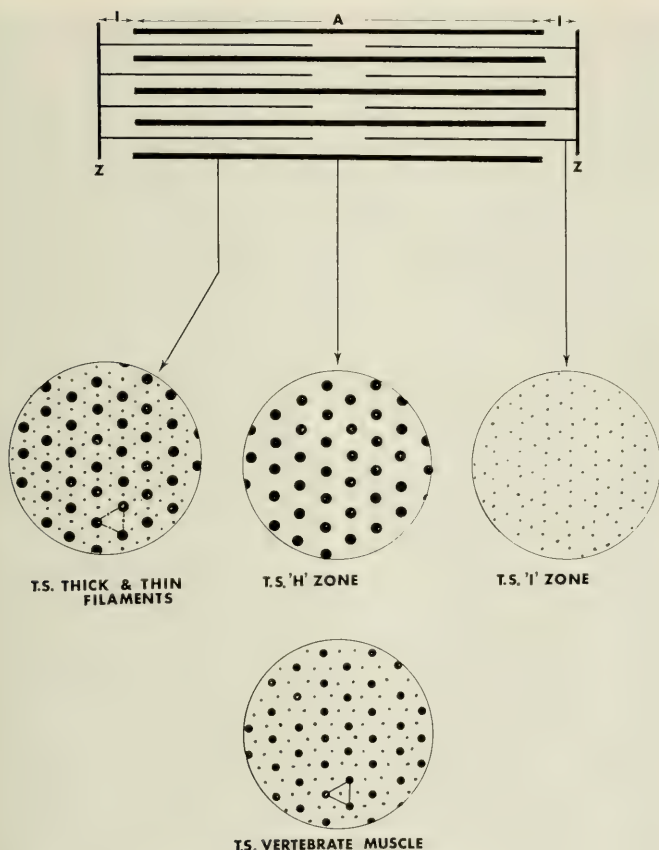


FIGURE 2. Diagrammatic representation of the myofilament arrangement in copepod muscle. Vertebrate muscle is also represented for comparison. If the myosin filaments in copepod muscle are imagined to be the apices of an equilateral triangle, then the actin filaments occur in the center of each side. In vertebrate muscle each actin filament lies equidistant from three myosin filaments.

existence of a continuous system of sarcoplasmic reticulum, leading from the muscle surface and running between the muscle fibers, myofibrils and myofilaments.

The paired nature of some of the longitudinal tubules within the myofibrils seen in transverse section is attributed to elongate membranous vesicles that lie along

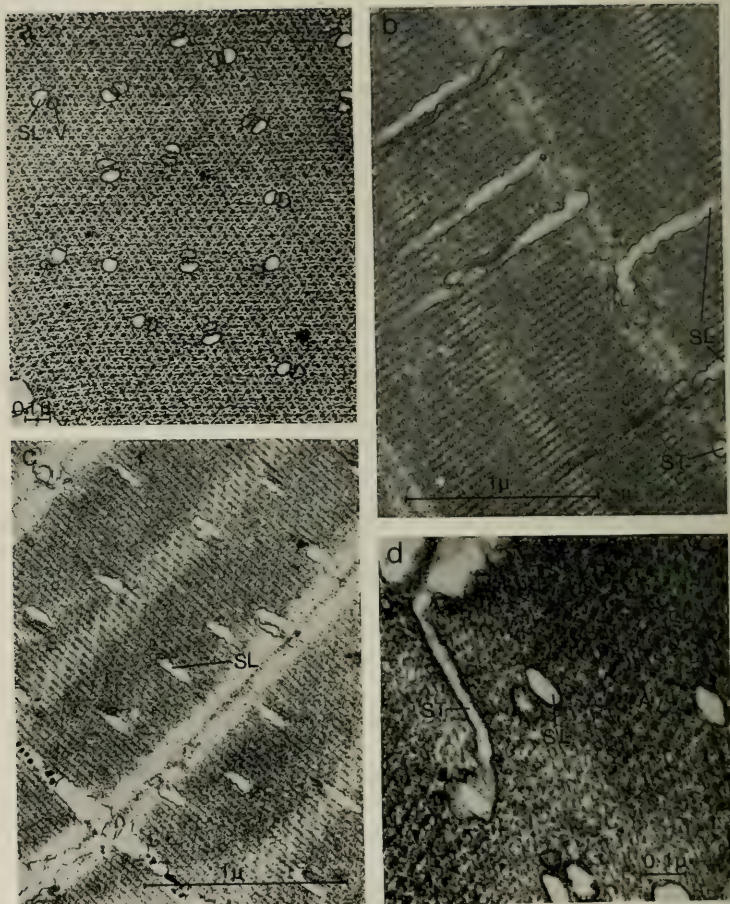


FIGURE 3. (a) Longitudinal sarcoplasmic reticulum elements (SL) and blind ending vesicles (V) in transverse section. The blind ending vesicles have a characteristic granular appearance to their lumen. (b) Longitudinal (SL) and transverse elements (ST) of sarcoplasmic reticulum as seen in longitudinal section. (c) Slightly oblique section through muscle showing longitudinal sarcoplasmic reticulum (SL) elements as oval vesicles. (d) Transverse section of *Paranthessius* muscle showing both longitudinal (SL) and transverse elements (ST) of sarcoplasmic reticulum in the I band (I). No transverse elements were observed in the A band (A).

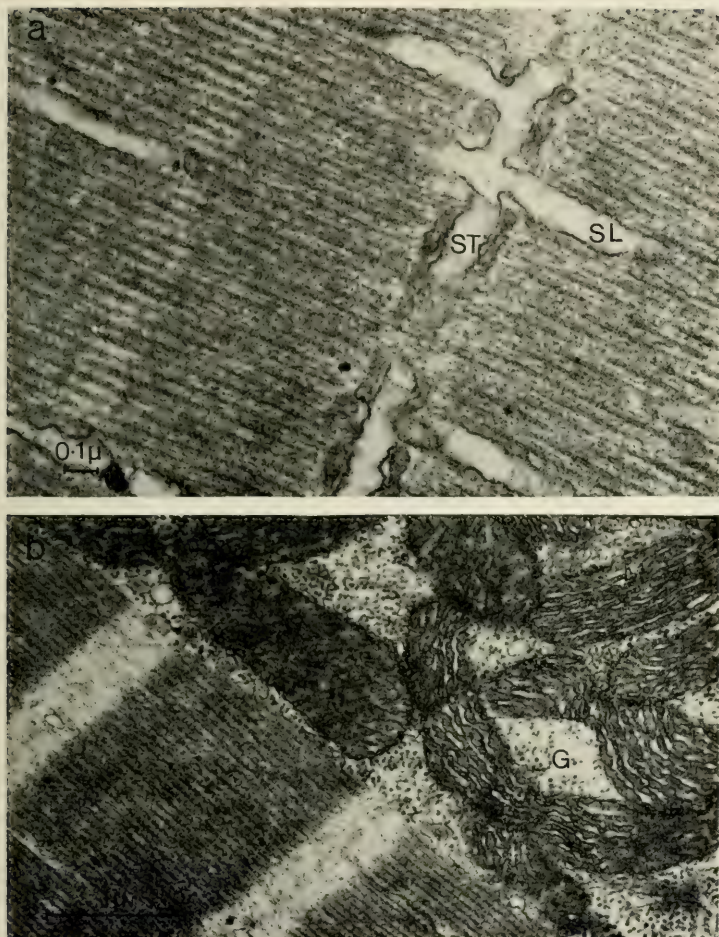


FIGURE 4. (a) Longitudinal section of *Paranthessius* muscle fibril showing junction of transverse sarcoplasmic reticulum (ST) and longitudinal sarcoplasmic reticulum (SL). (b) Mitochondria (M) and glycogen granules (G) on surface of muscle fibril.

longitudinal elements. The lumina of these vesicles have a granular appearance and their length rarely exceeds that of a sarcomere (Fig. 3a, 5). The general irregular nature and the occasional folding and branching of the longitudinal tubules

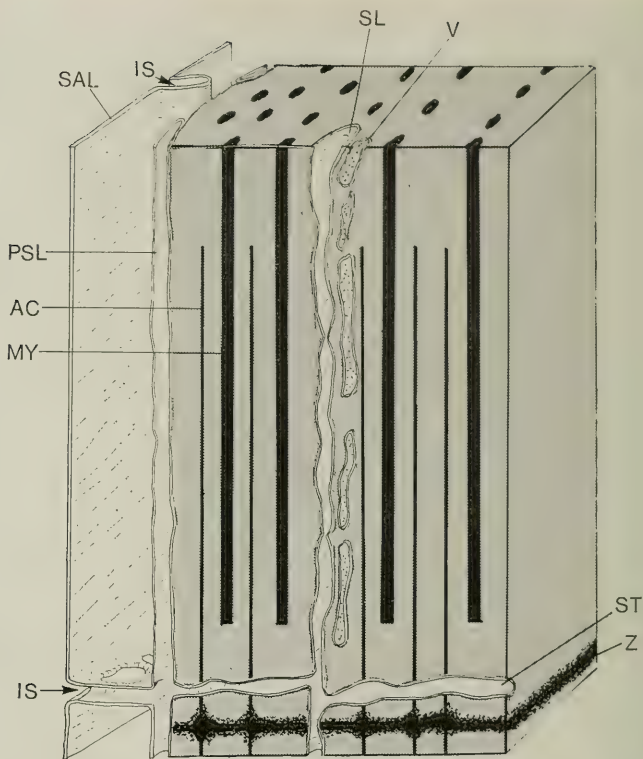


FIGURE 5. Three-dimensional diagram of the sarcoplasmic reticulum system in *Paramethessius anemoniae* muscle showing longitudinal sarcoplasmic reticulum (SL), vesicles (V), peripheral sarcoplasmic reticulum (PSL), transverse sarcoplasmic reticulum (ST), Z line (Z), actin filament (AC), myosin filament (MY), sarcolemma (SAL) and its invaginations (IS).

is probably accounted for by the occurrence of more than two units in some sections examined.

Scattered on the muscle surface under the sarcolemma are numerous mitochondria that may measure up to $2\ \mu$ in length, and $0.6\ \mu$ in width. The mitochondria of the muscle (or sarcosomes) are characterised by the possession of numerous cristae (Fig. 4b). Granules of glycogen (Fig. 4b) measuring about $25\ \text{nm}$ in diameter occur commonly between the myofibrils and are thought to be responsible for the strong positive reaction to the P.A.S. test for carbohydrates

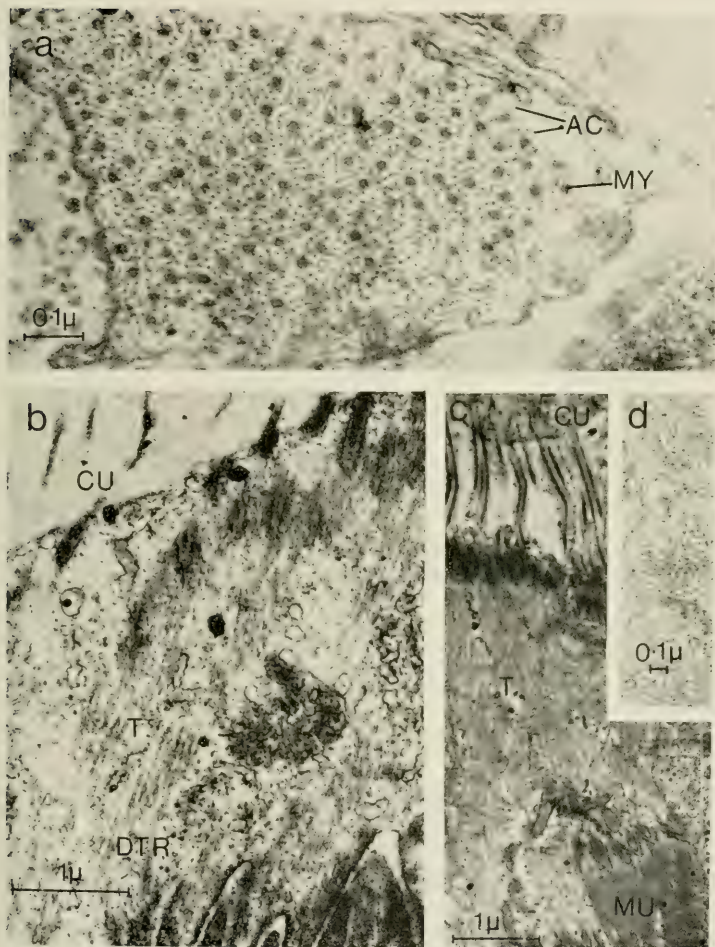


FIGURE 6. (a) Transverse section through muscle of alimentary canal showing both actin (AC) and myosin (MY) filaments. (b) Details of muscle junction with cuticle (CU) showing attachment fibrils (T) and dense terminal region (DTR) of muscle. (c) Further details of attachment of muscle (MU) to cuticle (CU) by tonofibrils (T). (d) Transverse section of the tonofibrils which attach muscle to cuticle in *Paranthessius*.

TABLE I

Dimensions of muscle fibrils in Paranthessius, Cyclops, Macrocylops and a vertebrate.

Species	Myosin			Actin thickness	Reference
	length	thickness	separation		
<i>Paranthessius</i>	1100 nm	12 nm	25 nm	4 nm	Bouligand (1964)
<i>Cyclops</i>	1500 nm	12 nm	25 nm	4 nm	
<i>Macrocylops</i>	—	15 nm	48 nm	—	Farenbach (1963)
Vertebrate	1500 nm	10 nm	45 nm	6 nm	Threadgold (1967)

observed in light microscope studies. The muscle nuclei are a flattened ovoid shape and have a single nucleolus. They are found in close contact with the muscle surface and were not commonly encountered during these investigations. The longitudinal muscle bundles and those supplying the appendages are constructed as described.

The muscle of the alimentary canal

The alimentary canal was found to be surrounded by muscle of a slightly different structure. There are several isolated bundles or strands of longitudinal muscle measuring 0.5 to 1.0 μ thick lying beneath the basement membrane of the digestive tract epithelium (Fig. 6a). Surrounding these longitudinal muscles is a layer of circular muscle of 1 μ average thickness. The myosin filaments of the gut muscle were seen in transverse section to be surrounded by 10 to 12 actin filaments instead of the six noted in the general body muscle. Although the myosin filaments are about the same distance apart as the general muscle (25 nm) they are somewhat thicker (15 nm). No internal ramification of sarcoplasmic reticulum is seen in this muscle.

The attachment of the muscle to the cuticle

Near the site of attachment to the cuticle the muscle fibrils terminate at an irregular electron-dense line that can be seen in longitudinal sections to traverse the fibril (Fig. 6b). More detailed examination, however, reveals that this electron-dense line is composed of the sarcolemma of the muscle, near to which, at a distance of 30 to 40 nm, is another plasma membrane that gives rise to fine tubules or tonofibrils (Fig. 6c, d). The tonofibrils measure about 20 nm in diameter and seem to pass, in groups, through thickened electron-dense zones, from each of which emerges an electron-dense fiber of roughly 400 nm thickness. These fibers ramify through the cuticle forming a firm attachment. The length of the tonofibrils ranges from 5 to 0.5 μ in different parts of the animal. Attachment is sometimes made to normal cuticle, while in other places the endocuticle invaginates to form an apophysis for muscle attachment (Briggs, 1978).

DISCUSSION

The observations made on *Paranthessius* muscle agree in many respects with those made by Bouligand (1962, 1963, 1964) on *Cyclops* and *Acanthocyclops*, by Fahrenbach (1963) on *Macrocylops* and by Raymont *et al.* (1974) on *Calanus*. In *Cyclops* the myosin filaments measure $1.5\ \mu$ in length compared to $1.1\ \mu$ in *Paranthessius*. *Macrocylops* has myosin filaments 15 nm thick, which are further apart than those of both *Cyclops* and *Paranthessius*. No detailed measurements are available for *Calanus* muscle. Dimensions of muscle structures given in Table I includes details of typical vertebrate muscle for comparison.

In a study of *Acanthocyclops*, Bouligand (1964) describes zones of double overlapping between the two sets of actin filaments of the sarcomeres when the muscle is contracted. A transverse section through this zone shows twice the number of actin filaments found in other regions of the muscle. In longitudinal sections of contracted muscle these overlapping zones or CM bands are characterised by appearing as a dark band across the muscle in the center of the H zone. Examination of many sections of *Paranthessius* muscle did not show this feature to be present. It is possible that the muscle examined here was fixed in the relaxed state. This is considered unlikely, however, since copepods always exhibited strong locomotory movements on encountering fixatives. The probability that all sections cut were of relaxed muscle is, therefore, very low. The general arrangement of the myofibrils is the same as that described for other copepods.

The sarcoplasmic reticulum in *Paranthessius* is very similar to that described by Bouligand (1962, 1963) for *Cyclops*, though it tends to be somewhat less elaborate than that studied by Fahrenbach (1962) in *Macrocylops*. In this species, the blind-ending membranous vesicles found in *Paranthessius* and also in *Cyclops* are more expanded, forming a well developed system of cisternae. As was found with the vesicles of *Paranthessius* muscle, the cisternae do not join with the elements of the sarcoplasmic reticulum, but come to within 10 nm in most regions of the muscle. The term dyad, used by Smith (1961), to describe the association between sarcoplasmic reticulum tubules and the cisternae in the beetle *Tenebrio* is used here to describe similar structures in copepod muscle. In *Paranthessius* a dyad represents the paired membranous tubules seen in a transverse section of muscle myofibrils. This is an association between a sarcoplasmic reticulum tubule and a blind ending vesicle.

The sarcoplasmic reticulum system of copepods may be contrasted with that of vertebrate muscle. In most vertebrates the sarcoplasmic reticulum forms a sleeve around the muscle fibril ending in a number of finger-like projections in the region of the Z line, where it comes into close contact with the invaginated membrane of the sarcolemma. This association forms a triad, composed of two sarcoplasmic reticulum elements (one from each side of the Z line of the fibril) and the membrane of the sarcolemma. The sarcolemma membrane is, therefore, not a continuous tubular system ramifying longitudinally through the myofibrils as is found in copepod muscle.

Fahrenbach (1963) stressed the importance of efficient diffusion of "transmitter substances" (Ca^{++}) in fast acting muscles. Slower contracting vertebrate muscles have the discontinuous triad structure described, in which the sarcolemma membrane

is not connected to the sarcoplasmic reticulum system. The myofilaments in the center of the myofibrils are not brought into such close proximity with a potential impulse-conducting element as is found in the arthropodan continuous dyad system of fast contracting muscle. In most fast muscle studied the distance that calcium ions have to diffuse in order to reach the center of the myofibrils to trigger contraction is maintained at a minimum distance of less than $1\ \mu$. Examples include 0.3 to $0.35\ \mu$ in the fast muscle of the dragon fly *Aeshna* (Smith, 1961), 0.18 to $0.2\ \mu$ in the toadfish *Opsanus* (Fawcett and Revel, 1961), 0.15 to $0.25\ \mu$ in the bat *Eptesicus* (Revel, 1962) and $0.2\ \mu$ in the copepod *Macrocylops* (Fahrenbach 1963). The value for *Paranthessius* was found to be on average $0.25\ \mu$. Fahrenbach (1963) proposes that this is the reason why the longitudinal tubules are arranged in a regular hexagonal manner in fast copepod muscle.

Bouligand (1962) suggests that the longitudinal sarcoplasmic reticulum elements of *Cyclops* are regularly arranged, so that their tendency to expand when the muscle contracts, (due to the hydrostatic pressure of their contained fluid) will not disorientate the myofilaments. Bouligand proposes that evidence for this may be gained from observation of true transverse sections of hexagonal array of the tubules. It is visualised that expansion force lines from these tubules would pass through the myosin filaments towards another expanding tubule, which would be exerting a similar force. This implies that the position of the myosin filaments would be undisturbed during muscle contraction. If the expansion forces tended to act between the myosin filaments the latter would be displaced to either side.

Both Fahrenbach's and Bouligand's interpretation of the regular arrangement of sarcoplasmic reticulum are applicable to *Paranthessius* muscle, which has a similar structure to that of the species studied by these authors. Although the sarcoplasmic reticulum is not so complex as that of *Macrocylops*, all other structural features indicate that the muscle of *Paranthessius* is of a fast contracting type. The muscles of the alimentary canal are probably slower acting, since they have nearly twice as many actin filaments as the general body muscles which is a characteristic of "slower" muscle (Fahrenbach 1967).

Parasitic forms usually have smooth or "slow contracting muscle" (Capart, 1948). It is noteworthy that *Paranthessius* has muscle characteristics of free-living forms, i.e., "fast acting muscle". This is not surprising when it is considered that *Paranthessius* is quite a mobile associated form (Briggs, 1974). It is of survival value for *Paranthessius* to be capable of rapid swimming in order to regain a position on its host if dislodged. Since the larval instars of *Paranthessius* live freely in the plankton, the infective stage must possess efficient locomotion for host location. The elaborate musculature of the adult may, therefore, represent a legacy from the free living phase of the life cycle.

Apart from the gripping claws of the second antenna and spinal reduction in the mouth parts, *Paranthessius* is relatively unmodified morphologically and bears a strong resemblance to free-living cyclopoids. It is, moreover, associated with the external surface of the host, never being found in the gastrovascular cavity, and is very mobile both on and off the anemone. These features together with the elaborate musculature described here suggest that in an evolutionary context *Paranthessius* is a recent invader of *Anemonia sulcata*. Comparative studies of the musculature in other associated species which exhibit varying degrees of host

dependence and morphological modification might add support to these speculations on the evolution of parasitism in copepods.

I wish to thank Professor Gareth Owen and Dr. R. V. Gotto of the Queen's University of Belfast for their invaluable encouragement and advice during this study, undertaken during the tenure of a postgraduate studentship from the Department of Education for Northern Ireland.

SUMMARY

1. *Paranthessius anemoniae* has striated muscle composed of actin and myosin myofilaments arranged hexagonally, as in free living copepods.
2. The sarcoplasmic reticulum is continuous with the membrane of the sarcolemma in the region of the Z line and forms a continuous system of tubules which ramify through the muscle.
3. Blind-ending vesicles form dyads with the longitudinal sarcoplasmic reticulum tubules.
4. Attachment of the muscle to the cuticle is by tonofibrils.
5. A relatively short sarcomere length, complex sarcoplasmic reticulum and high proportion of myosin to actin filaments indicate the "phasic" nature of the general body muscle in *Paranthessius*.
6. The muscle of the alimentary canal is characteristic of "tonic" muscle.

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RESPIRATORY ADAPTATIONS OF THE ESTUARINE MUD SHRIMP,
CALLIANASSA JAMAICENSE (SCHMITT, 1935) (CRUSTACEA,
DECAPODA, THALASSINIDEA)¹

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Thalassinid mud shrimps commonly burrow in hypoxic marine sediments, and their success in these habitats seems, at least in part, predicated upon metabolic adaptations. Recent studies of thalassinids from the Pacific coast of North America have identified a number of behavioral and physiological respiratory adaptations to the hypoxic habitats of intertidal species (Farley and Case, 1968; Thompson and Pritchard, 1969; Roxby, Miller, Blair, and Van Holde, 1974; Miller and Van Holde, 1974; Miller, Pritchard, and Rutledge, 1976; Torres, Gluck, and Childress, 1977; Hawkins, 1971, unpublished M.S. thesis, Oregon State University). A rich thalassinid fauna occurs in intertidal and sublittoral habitats along coasts of the western Atlantic, but metabolic regulation among these species has been investigated only in *Upogebia affinis* by Mangum and van Winkle (1973).

The present study concerns *Callianassa jamaicensis* (Schmitt) (*Callichirus jamaicensis* according to the generic scheme of de Saint Laurent, 1973), a common inhabitant of estuarine mud flats in the northern Gulf of Mexico (Felder, 1978) and other areas of the western Atlantic (Rodrigues, 1971). On the Louisiana coast, dense populations of *C. jamaicensis* are found in muddy substrates where low-salinity interstitial water is markedly hypoxic. Tidal exposure of these substrates frequently subjects such populations to extended periods of anoxia. Studies were undertaken to identify respiratory adaptations of *C. jamaicensis* to such hypoxic habitats. Specifically, this paper reports (i) survival under aquactic and aerial anoxia, (ii) aerial respiration, (iii) effects of oxygen tension on metabolic rate, and (iv) post-anoxia metabolic rates.

MATERIALS AND METHODS

Animals were collected from a tidally influenced pond on Grand Terre Island, Louisiana. Methods of collecting, transporting, maintaining and salinity-acclimating animals were the same as previously described (Felder, 1978). Animals were acclimated to a salinity of 20‰ in dark, 25° C incubators; all were maintained at this salinity for nine days before experiments were initiated. Animals were not fed, and aeration was provided during all phases of salinity-acclimation. Only intermolt, uninjured adult males were used in respiration studies. Wet weights were determined by thoroughly blotting animals with tissue and then weighing to the nearest milligram. All sea water used in experimental studies was carefully maintained at a salinity of $20 \pm 0.3\text{‰}$ and temperature of $25 \pm 0.2^\circ \text{C}$.

Anoxic sea water was prepared by gassing sea water with nitrogen. In one

¹ In part adapted from a doctoral dissertation submitted to the Department of Zoology and Physiology, Louisiana State University, Baton Rouge.

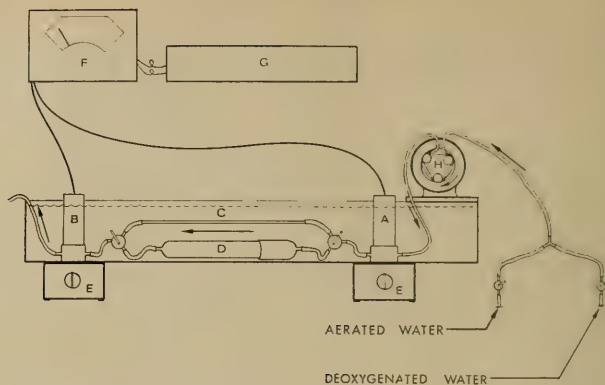


FIGURE 1. Diagrammatic cut-away view of water bath showing components of flow-through respirometer: A, influent oxygen electrode; B, effluent oxygen electrode; C, bypass shunt; D, respiration chamber; E, magnetic stirrer; F, differential oxygen meter; G, integrating chart recorder; H, peristaltic pump. A small bubble trap (not shown) was installed between components H and A.

experiment anoxic sea water was siphoned into BOD (300-ml biochemical oxygen demand) bottles containing one animal each, and bottles were sealed until death occurred. Other animals in individual, perforated vials were placed as a group into 5 liters of anoxic sea water which was replaced daily; whenever the 5-liter jar was opened for removal of dead animals, it was regassed with nitrogen. Control animals were maintained in continuously aerated sea water. Tolerance of aerial anoxia was determined by supporting animals on the rack of a desiccator over water and continuously gassing the water with nitrogen. Control animals in an aerial environment were likewise maintained, but underlying water was gassed with air.

Aerial V_{O_2} was measured in a Gilson respirometer with 130-ml respirometry flasks and equivalent ballast. Filter paper wicks and 30% KOH were added to each flask side-arm to absorb CO_2 . Each flask contained one animal and one milliliter of sea water to maintain water saturation of air. One hour was allowed for equilibration; thereafter oxygen consumption was read at 30-min intervals.

Oxygen consumption at decreasing oxygen tensions was measured by placing the animal into a 13.5-mm ID (inner diameter) plastic tube with openings at both ends. The tube was wedged vertically against the wall of a BOD bottle, and a small stirring bar was placed at the center of the bottle. An oxygen electrode was fitted snugly into the bottle opening, and depletion of oxygen was recorded with a Beckman oxygen analyzer. The analyzer was calibrated in air-saturated sea water and checked by Winkler titration (Strickland and Parsons, 1972) before each run. Temperature was maintained by a water bath supported over the magnetic stirrer. The stirring rate was set at the lowest speed, which produced maximum deflection of the oxygen meter. The displacement volume of the animal, tube, and stirring bar

was subtracted from the bottle volume. Pleopod ventilatory strokes were counted during 5-min intervals and expressed as mean number/min. To minimize effects of handling, each animal was transferred to a plastic tube and placed (anterior end up) into a BOD bottle 30 min before it was sealed; as an additional precaution, the first 30 min of recorded oxygen depletion were discarded.

A flow-through respirometer was assembled from a dual-probe International Biophysics differential oxygen analyzer, a Houston Instrument integrating chart recorder, a peristaltic pump, two magnetic stirrers, a constant temperature water bath, and a 16-mm ID glass respiration chamber (Fig. 1). Flow rate was maintained at *ca.* 10 ml/min and was precisely determined by measuring the volume of effluent; injection of a dye at this flow rate indicated thorough mixing of water as it passed through the respiration chamber. Each animal was placed into the chamber with its anterior toward the influent opening; aerated water was provided for 1 hr before oxygen consumption was read. Prior to each run, the oxygen analyzer was calibrated by Winkler titration and the differential between the electrodes was set to zero. Altered oxygen tensions were achieved by controlled mixing of fully aerated and nitrogen-saturated water. Whenever oxygen content of influent water was altered, 20 min were allowed for the flow-through system to flush before oxygen consumption was read. Anoxic conditions were provided by pumping deoxygenated water into the respiration chamber and then closing valves at either end (Fig. 1). The flow-through respiration chamber was lined with fine-mesh plastic gauze to provide traction for thoracic legs during pleopod beating. Pleopod strokes were counted as previously described.

Field measurements of oxygen in exposed and submerged burrows were made during an afternoon low tide in July 1972. Oxygen concentration of water over-

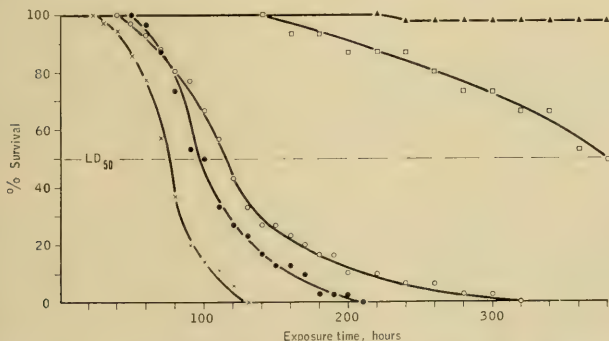


FIGURE 2. Survival among specimens of *Callianassa jamaicensis* under aerial and aquatic anoxia compared to survival of controls under normoxia. Experimental conditions include anoxic water with accumulation of metabolic wastes (crosses) ($N = 35$), anoxic water changed daily (solid circles) ($N = 30$), aerial anoxia (open circles) ($N = 30$), aerial normoxia (squares) ($N = 15$), and aquatic normoxia (triangles) ($N = 50$); N is the number of animals initially exposed to each condition. Temperature was maintained at $25 \pm 0.2^\circ \text{C}$; salinity was $20 \pm 0.3\text{‰}$.

lying *C. jamaicense* burrows was measured *in situ* with an air-calibrated Yellow Springs Instrument oxygen meter. Water from burrows of *C. jamaicense* was sampled and analyzed as described by Thompson and Pritchard (1969).

RESULTS

Survival under anoxia

Survival of specimens of *C. jamaicense* under aquatic and aerial anoxia is plotted in Figure 2. The LD₅₀ (mean lethal dose) was lowest, *ca.* 3.2 days, when anoxic water was not changed and metabolic wastes accumulated for the duration of survival. Under such conditions, with individual animals sealed into 300-ml BOD bottles of anoxic water, ambient pH dropped from an initial level of 8.7 ± 0.3 to 6.9 ± 0.5 at the time of death. When anoxic water was replaced daily, the LD₅₀ increased to *ca.* 4 days. The LD₅₀ for animals held in aerial anoxia, *ca.* 5 days, exceeded that for animals subjected to aquatic anoxia. Among those animals subjected to aerial anoxia or daily changes of anoxic water, a few survived more than two times the exposures producing LD₅₀'s.

Under normoxic conditions, losses of control animals in a water-saturated environment approached the LD₅₀ on the 16th day of exposure. Mortalities among control animals in normoxic water did not exceed 2% within the same 16-day time period.

Aerial oxygen consumption

After 60 min of equilibration in a 25° C Gilson respirometer, oxygen consumption ($\dot{V}_{O_2}$) in water-saturated air was read at 30-min intervals over an additional 2-hr period. Wet weights of the 25 animals used in the aerial respiration experiments ranged from 3.51 to 5.25 g. Mean $\dot{V}_{O_2}$ rates and standard errors over the four successive 30-min time periods were 18.9 ± 1.53 , 16.2 ± 1.29 , 19.0 ± 1.39 , and 18.9 ± 1.12 $\mu\text{l}/(\text{g wet wt}\cdot\text{hr})$, respectively. Activity in the respirometer flasks was not quantitatively monitored, but animals were for the most part quiescent during the $\dot{V}_{O_2}$ determinations.

Effects of low oxygen tension on aquatic oxygen consumption

As oxygen was depleted from sealed BOD bottles, specimens of *C. jamaicense* regulated $\dot{V}_{O_2}$ until oxygen tension (P_{O_2}) decreased to *ca.* 20 mmHg (Fig. 3). The critical oxygen tension (P_c) ranged from 10 to 25 mmHg among the 10 animals studied. The slightly higher $\dot{V}_{O_2}$ at 120 mmHg is of questionable significance as it may relate to disturbance of animals when placing them into the BOD bottles at the beginning of the experiment. Mean pleopod ventilatory rates ranged from 20 to 33 strokes/min at oxygen tensions above the P_c . As P_{O_2} fell from 20 to 10 mmHg, pleopod activity increased to near 60 strokes/min; concurrent increases in $\dot{V}_{O_2}$ occurred in some animals and accounted for the large range of $\dot{V}_{O_2}$ at oxygen tensions between 12 and 15 mmHg (Fig. 3). Pleopod activity decreased as P_{O_2} dropped below 9 mmHg and was again near 39 strokes/

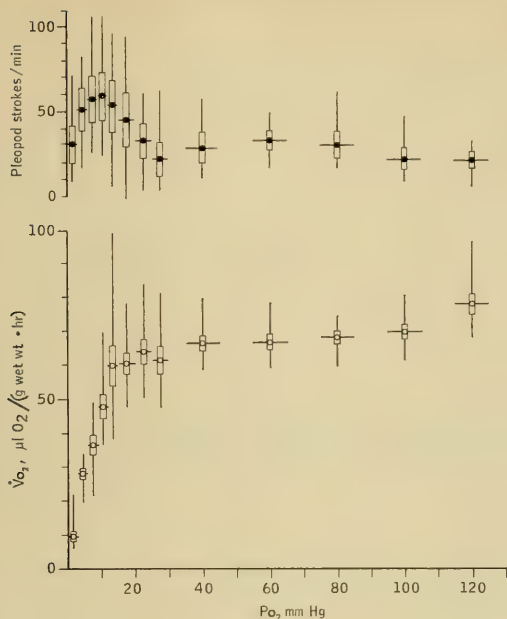


FIGURE 3. Mean oxygen consumption (open circles) and mean ventilatory rate (solid circles) among specimens of *Callianassa jamaicensis* as oxygen is depleted from a sealed bottle. Each open circle is mean value for 10 animals and each solid circle is mean value for eight animals. Vertical lines indicate ranges; rectangles indicate standard errors; horizontal lines indicate span of oxygen tension over which means are taken. Wet weights range from 1.45 to 3.82 g. Temperature was maintained at $25 \pm 0.2^\circ \text{C}$; salinity was $20 \pm 0.3\text{‰}$.

min at 0 mmHg. Animals held at complete anoxia continued to decrease pleopod activity and, after 2 to 4 hr, stopped ventilating unless disturbed.

When P_{O_2} was abruptly decreased from normoxia (150 mmHg) to hypoxia (37 mmHg) in a flow-through respirometer, specimens of *C. jamaicensis* reduced $\dot{V}_{O_2}$ by more than 50% for 2 to 3 hr (Fig. 4). Oxygen consumption gradually increased after 5 hr of hypoxia and after 9 hr was near 75% of $\dot{V}_{O_2}$ in normoxia. In normoxia, pleopod activity ranged from 14 to 18 strokes/min. As P_{O_2} decreased to 37 mmHg, pleopod activity at first increased slightly but soon subsided to rates less than those in normoxia.

When introduction of hypoxic water (37 mmHg) followed 12 hr of anoxia, the $\dot{V}_{O_2}$ of *C. jamaicensis* was initially just above that observed under normoxia (Fig. 5). The $\dot{V}_{O_2}$ in hypoxia decreased slowly from the rates measured shortly after termination of anoxia. Pleopod activity, which was negligible during anoxia,

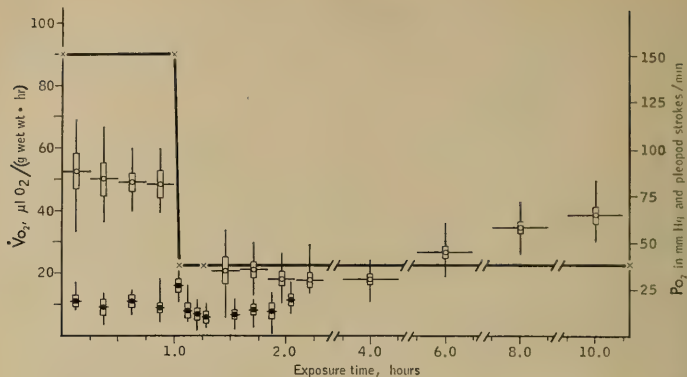


FIGURE 4. Temporal variations in aquatic oxygen consumption (open circles) and pleopod ventilatory rate (solid circles) among specimens of *Callinassa jamaicensis* when ambient oxygen tension (crosses on heavy line) is abruptly reduced. Each value is mean rate for five animals. Vertical lines indicate ranges; rectangles indicate standard errors; horizontal lines indicate time spans over which means are taken. Temperature was maintained at $25 \pm 0.2^\circ \text{C}$; salinity was $20 \pm 0.3\text{‰}$.

dramatically increased with introduction of hypoxic water. As hypoxic water entered the respiration chamber following 12 hr of anoxia, animals invariably moved to the influent opening of the chamber (Fig. 1) and began rapid ventilation with their pleopods. The accelerated pleopod activity was maintained near 50 strokes/min for ca. 30 min after hypoxic water was introduced into the chamber, and animals spent almost all of this time near the influent opening of the respiration chamber. A gradual decrease of pleopod activity paralleled the slowly decreasing $\dot{V}O_2$ which began near the middle of hour 14 and continued through hour 15.

When anoxia was terminated by introducing normoxic (150 mmHg) water, the $\dot{V}O_2$ increased to two times the rates preceding anoxia (Fig. 6). Five hours after anoxia was terminated, $\dot{V}O_2$ approached that observed before anoxia. With the reintroduction of normoxic water, animals moved to the influent opening of the respiration chamber and rapidly ventilated with their pleopods as when hypoxia (37 mmHg) followed anoxia (Fig. 5). However, animals neither remained at the incurrent opening nor maintained accelerated pleopod activity for as long as when hypoxic water followed anoxia.

Field measurements of dissolved oxygen

Burrows of *C. jamaicensis* contained very low concentrations of dissolved oxygen when located above the waterline. On Grand Terre Island fluctuating tides exposed numerous burrows along pond margins for periods varying from a few hours to several days. Oxygen tension in water from five active burrows located from 1 to 3 m outside the pond ranged from 0 to 5 mmHg ($\bar{x} = 2.2 \text{ mmHg}$).

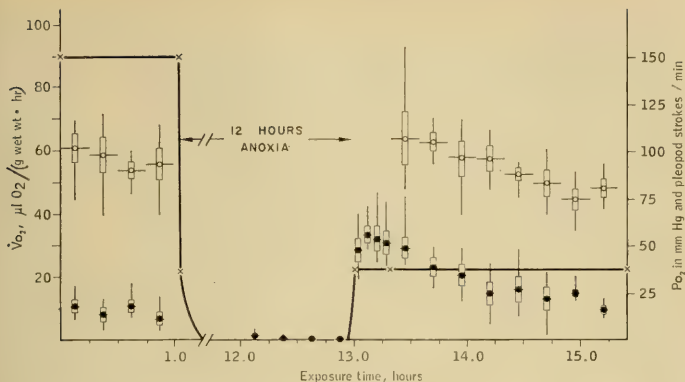


FIGURE 5. Temporal variations in aquatic consumption (open circles) and pleopod ventilatory rates (solid circles) among specimens of *Callianassa jamaicensis* when ambient oxygen tension (crosses on heavy line) is dropped to anoxia and then raised to hypoxia. Each value is mean rate for five animals. Vertical lines indicate ranges; rectangles indicate standard errors; horizontal lines indicate time span over which means are taken. Temperature was maintained at $25 \pm 0.2^\circ \text{C}$; salinity was $20 \pm 0.3\text{‰}$.

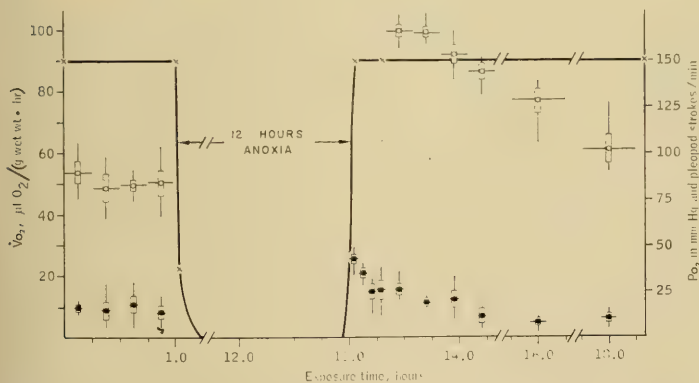


FIGURE 6. Temporal variations in aquatic consumption (open circles) and ventilatory rates (solid circles) among specimens of *Callianassa jamaicensis* when ambient oxygen tension (crosses on heavy line) is dropped to anoxia and returned to normoxia. Each value is mean rate for five animals. Vertical lines indicate ranges; rectangles indicate standard errors; horizontal lines indicate time spans over which means are taken. Temperature was maintained at $25 \pm 0.2^\circ \text{C}$; salinity was $20 \pm 0.3\text{‰}$.

which was well below critical oxygen tensions (P_c) established for specimens of *C. jamaicensis* in the laboratory. Longer periods of isolation from estuarine pond waters were caused by storm-effected movements of sand which elevated large areas of the pond above water level, sometimes for periods of several months. Excavation of one such area which had been isolated from the pond for 2 months produced numerous living specimens of *C. jamaicensis*, although most were moribund; some of these moribund specimens appeared to be occupying portions of burrows above the water table.

Higher and more variable oxygen tensions occurred in burrows at the immediate edge of the pond and just inside the pond. Oxygen tensions in 10 of these burrows, located from 0.2 to 2.0 m inside the pond, ranged from 11 to 119 mmHg ($\bar{x}$ = 73.4 mmHg). However, at the time of sampling, oxygen tensions in surface waters were from 151 to 161 mmHg, or well above concentrations in burrows. Oxygen tensions in water of the estuarine pond probably approached these levels only during the periods of photosynthetic activity. Diel cycles of oxygen in water overlying *C. jamaicensis* burrows on Grand Terre Island were characterized by decreasing tensions after dark as photosynthesis was replaced by net community oxygen consumption. Diel variations were monitored in October, 1974 (J. Day, personal communication), and P_{O_2} of water overlying *C. jamaicensis* burrows remained below the P_c of *C. jamaicensis* during a 4 to 5 hr period just before to just after dawn.

DISCUSSION

The limited studies available to von Brand (1946) led him to conclude that decapod crustaceans show little tolerance of anoxia. Extended tolerance of anoxia is, however, one adaptation exhibited by a number of decapods which burrow in potentially hypoxic substrates. For instance, mud-burrowing crayfish survive anoxia four times longer than those inhabiting swift streams (Bovbjerg, 1952). Among the thalassinid decapods, *Upogebia pugettensis* and *Callinassa californiensis* survive anoxia for at least three days (Thompson and Pritchard, 1969) and *Callinassa jamaicensis* survives anoxia for three to four days (Fig. 2). These and other thalassinids, such as *Callinassa affinis* in Southern California (Congleton, 1974) and *Callichirus foresti* from west Africa (LeLoeuff and Intes, 1974), are highly specialized for a burrowing existence in shallow, hypoxic, marine substrates, and their tolerance of anoxia is clearly an adaptation to habitat.

Under aquatic anoxia, the longer survival of specimens of *C. jamaicensis* when anoxic water is changed daily, compared to survival of specimens when anoxic water is not changed, may reflect the effects of accumulated metabolic wastes. Products of anaerobic metabolism could account for the decrease in pH observed when individuals of *C. jamaicensis* are sealed into BOD bottles of anoxic water and left until death occurs. The buffering characteristics of burrow water, particularly in the lower-salinity extremes of *C. jamaicensis* habitat, or the ability to exchange anoxic burrow water might thus affect survival of *C. jamaicensis* when it is subjected to periods of anoxia in nature. Short-term survival of *C. jamaicensis* does not, however, appear to be affected by the physical presence or absence of the burrow, although MacGinitie (1934) reports that a specimen of *Callinassa*

californiensis will soon die if not maintained with its body contacting the wall of a tube. In either anoxia or normoxia, survival of specimens of *C. jamaicense* does not seem to be influenced by whether animals are maintained in plastic tubes or individually in larger bottles and open dishes. The possibility remains, however, that the tube facilitates efficient respiration in hypoxic water.

Survival and oxygen consumption by specimens of *C. jamaicense* in air were investigated despite the lack of direct evidence that this species resorts to aerial respiration in nature. However, on several occasions living animals were collected from substrates which had been exposed for up to 2 months, and on one occasion they were collected from a mud bank more than 1 m above the water table. As negligible concentrations of oxygen are usually found in water of exposed burrows, and as *C. jamaicense* has the ability to survive (Fig. 2) and respire in water-saturated air, occupancy of exposed upper portions of the burrows seems at least a plausible alternative to longterm anoxia. Aerial respiration is best documented among terrestrial and semiterrestrial decapods but is also used on an "emergency" basis by a number of aquatic species (Wolvekamp and Waterman, 1960). For example, *Carcinus maenas* may raise its body and aerially ventilate when stranded in hypoxic ponds (Taylor and Butler, 1973). The humid environment of *Callianassa* burrows fulfills an important requirement for aerial respiration as oxygen diffuses most rapidly across a wet cuticle (Lockwood, 1967).

The lower $\dot{V}_{O_2}$ of *C. jamaicense* in saturated air than in water could be attributed in part to decreased activity under aerial conditions as high P_{O_2} is maintained at the respiratory surface without the need for extensive ventilatory movements. The aerial $\dot{V}_{O_2}$ was less than 40% of aquatic $\dot{V}_{O_2}$ measured in BOD bottles or the flow-through system. This difference in aerial versus aquatic $\dot{V}_{O_2}$ is much greater than that reported for well-adapted semi-terrestrial decapods and suggests that the ability for aerial uptake of oxygen is not particularly well developed. It is not known to what degree *C. jamaicense* depends upon anaerobic pathways under such conditions as at least a partial source of energy. As suggested by Miller *et al.* (1976), it would be interesting to investigate the possible use of anaerobic pathways even at high oxygen tensions.

Within the P_{O_2} range of respiratory independence, $\dot{V}_{O_2}$ of *C. jamaicense* is ca 68 $\mu\text{l}/(\text{g wet wt}\cdot\text{hr})$ in a stirred BOD bottle from which oxygen is being depleted and 50 to 55 $\mu\text{l}/(\text{g wet wt}\cdot\text{hr})$ in a flow-through (10 ml min) respirometer. Both of these methods involve placement of the animal into a small diameter tube which simulates a burrow and allows the animal to brace itself while ventilating. Measurement of $\dot{V}_{O_2}$ in tubes seems to provide the better index of "routine" (*sensu* Fry, 1975) metabolic rates in burrowing thalassinids as this situation most closely approximates the natural mode of respiration. The reported respiration rates for intermolt specimens of *Callianassa californiensis* over the P_{O_2} range of respiratory independence are for animals not in tubes (Thompson and Pritchard, 1969; Miller *et al.*, 1976; Torres *et al.*, 1977), and these rates vary from ca. 18 to ca. 34 $\mu\text{l}/(\text{g wet wt}\cdot\text{hr})$. Farley and Case (1968) have shown that pleopod activity is clearly greater when a specimen of *Callianassa californiensis* is placed into a small diameter tube than when it is placed into a tube too large for it to brace against tube walls while countering pleopod strokes. In the present study smaller tubes were used in BOD bottles and this necessitated the

use of smaller animals (1.45–3.82 g) than in the flow-through respirometer (4.55–7.13 g); this size difference, as previously suggested by Torres *et al.* (1974), could account for observed differences in $\dot{V}_{O_2}$. Differences in the stirring or flowing of water during measurements of $\dot{V}_{O_2}$ in BOD bottles and the flow-through system may additionally contribute to a difference in $\dot{V}_{O_2}$ measured by those methods, as flow characteristics can engender adaptive respiratory responses (Mangum and van Winkle, 1973).

Regardless of the method of measurement, the metabolic rates here reported for *C. jamaicense* rank among the lower known for crustaceans at similar temperatures (Wolvekamp and Waterman, 1960) and reflect metabolic adaptation to a hypoxic habitat. Low metabolic rates in *Callianassa californiensis* and *Upogebia pugettensis* at 10° C are also considered adaptations to a similarly hypoxic habitat (Thompson and Pritchard, 1969). Montuori (1913) reports a much higher $\dot{V}_{O_2}$ of 132 $\mu\text{l}/(\text{g wet wt}\cdot\text{hr})$ in *Callianassa subterranea* and 368 $\mu\text{l}/(\text{g wet wt}\cdot\text{hr})$ in *Gebia littoralis* (*Upogebia littoralis*) at 25° C, but experimental conditions of his study differ too greatly to permit detailed comparisons of data.

The low critical oxygen tension (P_c) for *Callianassa jamaicense* likewise suggests a metabolic adaptation. Metabolic regulation is common to a large number of aquatic crustaceans, and van Winkle and Mangum (1975) note that such regulation is expected where the path of oxygen permeation is restricted to an indirect route by way of circulating body fluids. The P_c between 10 and 25 mmHg for *C. jamaicense* (Fig. 3), like that reported for other thalassinids (Thompson and Pritchard, 1969; Miller *et al.*, 1976; Torres *et al.*, 1977) is well below the P_c for most crustaceans (Wolvekamp and Waterman, 1960). Hypothetical curves of oxygen consumption over decreasing P_{O_2} , as predicted by a polynomial model (Mangum and van Winkle, 1973), suggest a P_c for *Upogebia affinis* similar to that reported for *U. pugettensis* but not as low as those in *Callianassa californiensis* or *C. jamaicense*. It has been suggested that the P_c of crustaceans represents the P_{O_2} at which blood pigment fails to become saturated at the gills (Redmond, 1955) or, more recently, that it reflects the initiation of anaerobiosis (Young, 1973). Regardless, maintenance of aerobic respiration until a very low P_{O_2} is reached would seem a conservative adaptation for burrowers in hypoxic substrates.

Present data do not explain the mechanics involved in metabolic regulation by *Callianassa jamaicense* at low P_{O_2} . Neither pleopod activity (Fig. 3) nor heart rate (Thompson and Pritchard, 1969) shows a linear increase with decreasing ambient P_{O_2} . The findings of Torres *et al.* (1977) additionally show that complete immobilization of the pleopods causes no appreciable change in the P_c . However, scaphognathite ventilation rates were not monitored, and it remains to be seen whether scaphognathite ventilation rate, cardiac output, and circulation patterns undergo proportional increases at lowered P_{O_2} . Studies with totally bled specimens of *Callianassa* suggest that at least part of the ability to regulate $\dot{V}_{O_2}$ is due to respiratory properties of the blood itself (Miller *et al.*, 1976).

The increase in pleopod activity at and just below the P_c may reflect an "escape" reaction; escape from low P_{O_2} in nature could be achieved by rapid pleopod ventilation which would replace low P_{O_2} burrow water with higher P_{O_2} water from overhead. Periodic ventilatory pulses, such as those reported for *Callianassa filholi* when specimens are confined to a glass tube (Devine, 1966), may likewise

be such reactions triggered by depletion of oxygen to a concentration near the P_{O_2} . Such reactions and taxic responses exhibited during flow-through respirometry suggest the presence of an internal or external oxygen receptor in *C. jamaicense*; Farley and Case (1968) have previously postulated the existence of such a receptor in *Callianassa californiensis* and *C. affinis*, but direct evidence for an oxygen receptor is still lacking for any thalassinid species.

The drop in $\dot{V}_{O_2}$ following abrupt exposure of specimens of *Callianassa jamaicense* to hypoxic water (Fig. 4) indicates that regulation of metabolic rates in low P_{O_2} is dependent upon how fast hypoxia is approached. The decrease in $\dot{V}_{O_2}$ suggests a partial shutdown of aerobic respiration or loss of metabolic regulatory ability unless hypoxia is approached slowly. Mangum (1970) reports aerobic shutdown in bloodworms rapidly introduced into hypoxic water, and Kushins and Mangum (1971) note that metabolic response of the snail, *Nassarius*, depends upon how rapidly hypoxia is approached. Similarly, Hiestand (1931) reports that a crayfish which normally responds as a metabolic regulator will metabolically conform if placed into a small volume of water where P_{O_2} is reduced rapidly or if depletion of oxygen in a large jar commences at less than air saturation. Because $\dot{V}_{O_2}$ of *C. jamaicense* slowly increases after several hours in hypoxic water (Fig. 4), it appears that a time-dependent internal change, such as decrease in pH or re-establishment of diffusion gradients, is linked to ability to regulate $\dot{V}_{O_2}$. This suggests that some degree of low- P_{O_2} acclimation is induced in *C. jamaicense* after six or more hours of hypoxia.

The lowest ambient oxygen tensions in the *Callianassa jamaicense* habitat occur on occasions when burrows are exposed and animals cannot ventilate by pumping water from overhead. An increase in $\dot{V}_{O_2}$ after termination of anoxia (Fig. 6) suggests the development of an oxygen debt under such conditions; a similar compensatory increase occurs in *Callianassa californiensis* and *Upogebia pugettensis* after exposure to anoxia (Thompson and Pritchard, 1969). Although evidence of oxygen debts among crustaceans is meager (Lockwood, 1967), clear evidence of anaerobic glycolysis in *Callianassa californiensis* tends to support this hypothesis (Hawkins, 1971, unpublished M. S. thesis, Oregon State University). Published field observations of several Pacific coast thalassinids (MacGinitie, 1935) and present observations of *Callianassa jamaicense*, *C. major*, and *C. islagrande* on the Louisiana coast indicate these animals move to the upper portions of the burrows as high tides flood burrows exposed earlier by low tides. Such behavior would facilitate the most rapid exchange of burrow water and payment of an oxygen debt developed during anaerobiosis. The magnitude and duration of elevated $\dot{V}_{O_2}$ rates in *C. jamaicense* following anoxia are determined by the ambient P_{O_2} provided at the termination of anoxia, as evident in comparing Figures 5 and 6. The rate of oxygen uptake under such circumstances is at least passively affected by the blood-to-water gradient of P_{O_2} , and a pattern for metabolic regulation is temporarily supplanted by metabolic conformation and higher respiratory rates in normoxia. It seems very unlikely that the observed differences in post-anoxia $\dot{V}_{O_2}$ rates can be attributed to activity, because activity is greater and maintained at elevated rates for a longer period during slightly elevated $\dot{V}_{O_2}$ in hypoxic water following anoxia (Fig. 5) than during the greatly elevated $\dot{V}_{O_2}$ in normoxic water following anoxia (Fig. 6).

Oxygen uptake via areas of the integument other than gills has not been investigated in *C. jamaicense*, but as these animals have a thin exoskeleton, such extrabranchial uptake of oxygen seems a strong possibility and could prove advantageous in a hypoxic habitat. Although there is no conspicuous morphological evidence of specialized, extrabranchial respiratory surfaces in *C. jamaicense*, accessory pleopodal gill filaments occur on some thalassinids (de Saint Laurent, 1973). The experiments of Torres *et al.* (1977) show no evidence of extrabranchial uptake in pleopods of *Callinassa californiensis*, but further investigations of accessory uptake are warranted; such studies could possibly explain the low oxygen gradients between prebranchial and postbranchial blood reported by Miller *et al.* (1976).

SUMMARY

Callinassa jamaicense survives exposure to aquatic and aerial anoxia for more than 3 days. In normoxic water-saturated air it survives for *ca.* 16 days. The rate of oxygen consumption ($\dot{V}_{O_2}$) in air is less than 40% of $\dot{V}_{O_2}$ in water. Aquatic $\dot{V}_{O_2}$ is regulated above critical oxygen tensions (P_c) of 10 to 25 mmHg when animals are allowed to slowly deplete oxygen from a sealed bottle. Mean aquatic $\dot{V}_{O_2}$ of animals in a flow-through respirometer or in tubes placed into sealed BOD bottles ranges from 50 to 68 $\mu\text{l}/(\text{g wet wt}\cdot\text{hr})$ over oxygen tensions (P_{O_2}) above the P_c .

After a 12-hr exposure to anoxic water, $\dot{V}_{O_2}$ is not regulated; post-anoxia $\dot{V}_{O_2}$ in hypoxic water (37 mmHg) is initially less than $\dot{V}_{O_2}$ measured in normoxic water (150 mmHg) before exposure to anoxia; post-anoxia $\dot{V}_{O_2}$ in normoxic water is initially two times the pre-anoxia $\dot{V}_{O_2}$ and suggests the development of an oxygen debt during anoxia. When P_{O_2} of ambient water is abruptly dropped from 150 to 37 mmHg, specimens of *C. jamaicense* exhibit a partial shutdown of aerobic metabolism, but the $\dot{V}_{O_2}$ begins to recover after 6 hr in hypoxia.

When oxygen tension is slowly decreased, pleopod ventilation rate varies little as P_{O_2} changes from 120 to 20 mmHg. The pleopod ventilation rate increases as P_{O_2} falls 20 to 10 mmHg, but decreases below 10 mmHg and stops after several hours under anoxia. The rapid response of taxis and pleopod activity when *C. jamaicense* is exposed to altered P_{O_2} suggests rapid perception of external oxygen levels and provides further circumstantial evidence of an oxygen receptor in thalassinids.

Tolerance of anoxia, metabolic regulation to a low P_c , low metabolic rates, metabolic responses following anoxia, and taxic response to altered P_{O_2} constitute adaptations to the hypoxic habitat of *C. jamaicense*.

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AN ANALYSIS OF THE DEFENSIVE MECHANISMS OBSERVED IN
THE ANEMONE *ANTHOPLEURA ELEGANTISSIMA* IN
RESPONSE TO ITS NUDIBRANCH PREDATOR
AEOLIDIA PAPILLOSA

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The sea anemone *Anthopleura elegantissima* (Brandt, 1835) is a conspicuous member of mid-intertidal communities along the exposed rocky west coast of the United States (Hand, 1955; Ricketts and Calvin, 1962; Sebens, 1977). Several recent studies (Waters, 1973; Harris, 1973; Edmunds, Potts, Swinfin and Walters, 1975, 1976) have reported that *A. elegantissima* is a preferred prey of the anemone-eating aeolid nudibranch, *Aeolidia papillosa* (Linnaeus, 1767). Edmunds *et al.*, (1976) described behavioral reactions of *A. elegantissima* to attack, including bulging of the column at the site of attack, crawling, and releasing from the substrate. Howe and Sheikh (1975) characterized an alarm pheromone, anthopleurine, from *A. elegantissima* and described the behavioral response it elicited in the anemone. Howe and Harris (1978) demonstrated that *A. papillosa* acquires anthopleurine when feeding on *A. elegantissima* and that leakage of the pheromone caused the alarm response in other individuals. Waters (1973) speculated that *A. papillosa* is evolving to specialize on *A. elegantissima*.

Anthopleura elegantissima has well-developed behavioral responses to attack by *A. papillosa*, but none of these behaviors provides an effective defense in the laboratory. In addition, *A. papillosa* is consistently found associated with one of its least preferred prey, the subtidal anemone *Metridium senile* (Linnaeus, 1767) (Harris, 1973; Brewer, 1977). The ineffectiveness of the defenses of *A. elegantissima* against one of its chief predators under laboratory conditions and the fact that the predator is primarily associated with a less preferred prey suggests that the defenses may be more effective under natural conditions. The purpose of this study was to investigate a series of potential defensive mechanisms which may provide at least partial protection for *A. elegantissima* against *A. papillosa*. The effectiveness of these mechanisms were then evaluated in the context of the environment in which this predator-prey association is found.

MATERIALS AND METHODS

This study was conducted during the period of January to June 1976, though L.G.H. had been making observations on *A. papillosa* since 1964. The laboratory experiments and observations were conducted at the facilities of Hopkins Marine Laboratory, Pacific Grove, California. Field studies were done at the laboratory and at two nearby locations in Monterey Bay (36° 37' N, 121° 53' W).

Some of the specimens of *Anthopleura elegantissima* were collected from a rock

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outcropping on the east side of the laboratory and adjacent to a ramp for an old boat works (Site I). These rocks face east and are exposed to direct sunlight from sunrise to late afternoon (1500–1600 hr). Most of the specimens of *Anthopleura elegantissima* and some of the specimens of *Aeolidia papillosa* were collected from the rocky intertidal zone under abandoned sardine canneries (Site II) located adjacent to the laboratory; this site is shaded by the buildings and only receives direct sunlight from sunrise until about 1200 hr. Most of the specimens of *A. papillosa* were obtained from a subtidal site under Wharf No. 2 in Monterey (Site III). At this site, specimens of *A. papillosa* of various sizes can be obtained throughout the year, feeding on the large concentrations of *Metridium senile* (Yarnall, 1972; Brewer, 1977). Animals were maintained in running sea water at ambient temperatures (14° C).

It is important to distinguish between two forms of *Anthopleura elegantissima* present in Monterey Bay and farther south. The clonal form of *A. elegantissima* is restricted to the midtide zone, forms clones by binary fission on open rock surfaces, and seldom grows to a column diameter greater than 50 mm. A solitary form of *A. elegantissima* is found in the lower intertidal zone and to depths of 15 m; it attaches in cracks in the rock substrate and has not been observed to reproduce asexually. Individuals of this anemone may attain a column diameter of greater than 80 mm. In this study, we will refer only to the clonal form.

Intensive surveys of two rocky intertidal sites (Sites I and II) were undertaken in March and again in May, 1976, to determine densities of *A. papillosa* associated with *A. elegantissima*. Site II under the canneries faced northeast and received direct sunlight only during the morning hours. Small specimens of *A. papillosa* were common among clones of *A. elegantissima* at this site from January through March, 1976. Sampling was done using a 1.64-m quadrat to determine the relative density of *A. papillosa* compared to the density of *A. elegantissima*. No specimens of *A. papillosa* were ever found associated with clones of *A. elegantissima* at Site I, so no quadrat sampling was done there nor at Site II in June.

Three experiments were conducted to assess the importance of desiccation as an environmental stress on *A. papillosa*. In the first, six specimens of *A. papillosa* and six specimens of *A. elegantissima* were placed in stacking dishes without water for 6 hr. The experiment was done in the laboratory to approximate the time of exposure at neap low tide on a sunless day. In the second and third experiments, groups of *A. papillosa* were placed on rocks among clones of *A. elegantissima* immediately after the water had receded below the clones and were retrieved just prior to reflooding by the incoming tide. At the end of each experiment, animals were placed in dishes containing fresh sea water and held for 24 hr. Those animals which were moribund or unable to hold onto the glass dish after 24 hr were considered lost, while those which were attached and crawling normally were rated as surviving the low tide. The justification was that animals which were too weak to remain attached to the rock would be washed free by wave action. A nudibranch washed off a rock in the midtide may ultimately survive, but it is at least removed from the anemones on which it had been feeding. Controls were left in running sea water during the experiments.

Observations were made on feeding encounters between *A. elegantissima* and *A. papillosa* to determine the sequence of attack, the behavioral responses of *A.*

elegantissima and the preference of *A. papillosa* for particular regions of the anemone. In the first experiment, a number of anemones were removed from rocks and placed in a dish coated with silicone grease which prohibited their attachment. These anemones were left in running sea water for 24 hr and at the time of the experiment a majority of them were open. Fifty nudibranchs (body length 2–3 cm) were released into the dish and allowed to feed for 5 hours. To determine sites of attack by *A. papillosa*, the anemones were then relaxed in 7% MgCl (in fresh water) and surveyed for tissue damage which was obvious by direct observation. In a second experiment, approximately 100 specimens of *A. elegantissima* from a single clone were placed in a running sea-water table and groups of four to five nudibranchs were placed among the anemones; attacks were described and tabulated.

Observations showed that contact of any part of the nudibranch with the column of an anemone caused local swelling of the column at the site of contact. Preliminary experiments showed that the anemones were responding to *A. papillosa* mucus. A series of experiments were conducted to determine the site of receptivity of *A. elegantissima* to *A. papillosa* mucus, the duration of the response and the specificity of this response relative to other nudibranch species.

The tentacles or the column of the anemones were touched with mucus-covered or blank cotton swabs in the first experiment. In experiment two, mucus was obtained from the back or the foot of *A. papillosa*, from the aeolid *Hermisenda crassicornis* (Eschscholtz, 1831) and from the dorid nudibranch *Anisodoris nobilis* (Odhner, 1907). *A. nobilis*, which eats only sponges, served as a control in the second set of experiments. In all tests, nudibranchs were rubbed with a wet cotton swab which was then applied to a part of the body of the anemone. A separate swab was used for each anemone. The behavior of the anemones and duration of any response was described from observations made several times an hour.

RESULTS AND OBSERVATIONS

Qualitative sampling of a number of exposed rocky intertidal habitats along the California coast by both authors over several years showed that *A. papillosa* is found in association with clones of *A. elegantissima* throughout the year. The density of nudibranchs tends to be higher in the winter than in the summer, and a majority of the animals seen and/or collected were large—between 30 and 80 mm in length.

At certain times of the year, *A. papillosa* can be very common in association with *A. elegantissima* (Table I). Small specimens of *A. papillosa* (mean length 12 mm; maximum 22 mm) were prevalent at the canneries site (Site II) from January through March. However, the mean length of animals observed and/or collected did not increase over this 3-month period, and they were not present a few hundred meters away at Site I. In late April, a storm pounded the intertidal zone with approximately 2.5-m waves. From late April through July no specimens of *A. papillosa* were observed at either of the intertidal stations, although at Site III all sizes were common in subtidal fouling communities (Table I). *A. papillosa* grows from 1 mm to over 30 mm in about 2 months when feeding on *A. elegantissima* in the laboratory (Harris, in preparation); this suggests that

TABLE I

Comparison of *Aeolidia papillosa* density in association with anemone clones at two intertidal and one subtidal (8 m) sites in Monterey Bay in winter and summer. Site I is a rocky outcropping on the beach on the NE side of Hopkins Marine Station. Site II is a rocky ledge under the canneries about 300 m E of Site I. Site III is under commercial Wharf No. 2 in Monterey. Site I and II contain clones of *Anthopleura elegantissima* and Site III is dominated by clones of *Metridium senile*. Where no quadrat numbers are given, a minimum of 5 hr of observations were made at each site during that month.

Number <i>A. papillosa</i> /m ² of anemone clone				
Site	February 1976	April 1976	June 1976	Time of direct exposure to sunlight
I	0/m ²	0	0	sunrise to ~1600.
II	24.3/m ² (104 1/64 m ²)	0	0	sunrise to ~1200.
III	86/m ² (10 1/10 m ²)	present	9.5/m ² (20 1/10 m ²)	none

nudibranchs were continuously recruiting to the site under the canneries but that they were not surviving long enough to reach sexual maturity.

Small specimens of *A. papillosa* (< 15 mm length) were typically found within 2 cm of the anemones. Often this was at the edge of a clone or within a scattered aggregate of anemones. A nudibranch might be in a small depression or crack in the rock surface, but the majority of individuals were exposed on flat surfaces, apparently having made no attempt to hide when the tide receded. Large specimens of *A. papillosa* (> 15 mm) may be found farther from their prey and typically seek out cracks in which to hide when not feeding. However, even with intensive

TABLE II

A summary of quadrat analyses done to assess the relative density of *Aeolidia papillosa* in relation to the percentage of free space among *Anthopleura elegantissima* clones. The sampling was done at Station II under the canneries on 12 February 1976. The quadrat size was 1/64 m², and 104 quadrats were sampled.

% Free space	0%	25%	50%	75%	Total
Number of quadrats	22	39	31	12	104
Number of quadrats with <i>A. papillosa</i>	2	17	11	4	34
Number of <i>A. papillosa</i>	2	18	18	5	44
					Mean
Number of <i>A. papillosa</i> per quadrat	0.09 ¹	0.46	0.56	0.41	0.42
Density of <i>A. papillosa</i> per m ² of <i>A. elegantissima</i> clone	5.8	29.4	35.8	26.2	24.3
% Quadrats with <i>A. papillosa</i>	9%	43%	35%	33%	33%

¹ Significant at <0.01 (*t*-test).

TABLE III

Results of desiccation experiments in which *Aeolidia papillosa* was exposed to air for 6 hr and then returned to fresh sea water. Survival was determined after 24 hr. Experiment 1 was done in the laboratory while experiments 2 and 3 were conducted in the field by placing nudibranchs adjacent to clones of *Anthopleura elegantissima* on the receding tide and retrieving them just prior to submergence on the incoming tide. The results were significant at less than 0.01% using chi square.

Experiment	1	2	3A	3B
Number of <i>A. papillosa</i>	6	10	32	33
Number surviving after 24 hr (%)	4 (66%)	4 (40%)	16 (50%)	13 (39%) ¹
Control survival	100%	100%	100%	100%

¹ Eighteen nudibranchs disappeared when the tide washed over the rock containing the animals before they could be retrieved; only 15 animals remained and the 18 missing *A. papillosa* were considered killed.

searching, no nudibranchs larger than 22 mm were found at Site II during the period from January through June 1976.

Results of the quadrat sampling at Site II illustrate the tendency of small nudibranchs to be found in open areas adjacent to groups of *A. elegantissima* (Table II). The quadrats were only placed over areas containing *A. elegantissima*, because numerous observations indicated that *A. papillosa* remains close to its prey. Only two nudibranchs were found within what appeared to be solid masses of anemones while the vast majority occurred at the periphery of clones. Close examination revealed that even in the most tightly packed clones, there was usually bare rock between pedal disks. The sampling data suggest that anemones within tightly packed clones are essentially free from predation by *A. papillosa*. While *A. papillosa* tended to be found at the periphery of clones where there is more free space, two other predators or parasites, the prosobranch *Epitonium tinctorum* (Carpenter, 1864) and the pycnogonid *Pycnogonum stearnsi* (Ives, 1892) were common only within these tightly packed aggregations.

While no individuals of *A. papillosa* were collected at Sites I and II during the late spring and summer of 1976, one of us (L.G.H.) had previously collected specimens of *A. papillosa* and egg masses at several locations (Eagle Point, San Juan Islands, Washington; Dillon Beach and Bodega Bay, California) during the months of June and July. These sites are more exposed open coastal locations than the protected environment of Monterey Bay. In each case, the density of *A. papillosa* was well below 1/m² of *A. elegantissima* clone. The majority of the nudibranchs were large (> 40 mm), sexually mature, and were located in tidepools or at the lower end of the anemone's distribution in the intertidal.

Results of tests on the effects of desiccation on *A. papillosa* are recorded in Table III. In both the laboratory and the field experiments, survival of test animals exposed to air for 6 hr was approximately 50%, while control animals maintained in running sea water had 100% survival. In the initial laboratory test, two animals died even though they were sitting in small amounts of residual water and mucus. Most of the nudibranchs were still reactive after 6 hr out of water, but those that ultimately died were unable to hold onto the dish after water was added.

Two smaller nudibranchs (about 20 mm) set out at Site I were so desiccated that they had to be scraped from the rock surface.

In Experiment 3B, (see Table III), 33 specimens of *A. papillosa* were placed among a clone of *A. elegantissima* at Site II. Due to a miscalculation, the nudibranchs were not retrieved until after a few small swells had already washed over the area. Only 15 out of the 33 animals were still attached to the rocks; the other

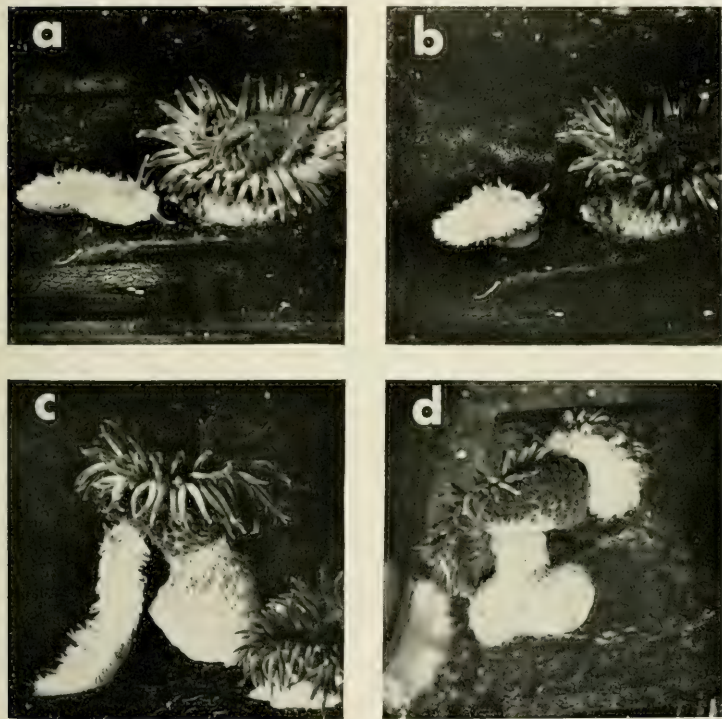


FIGURE 1. Photographic illustration of the initial contact between specimens of *A. papillosa* and *A. elegantissima* and two resulting feeding behaviors and anemone responses: (a) the initial contact involving the nudibranch's rhinophores and the anemone's tentacles; (b) the mutual retraction that typically occurs following first contact; (c) this nudibranch fed on the column for about 3 hr and made numerous attempts to reach the tentacles which were out of reach due to bulging of the column; (d) this nudibranch reached the tentacles before bulging of the column began and was lifted free of the substrate. The nudibranch fed in this position for about 3 hr; note line of mucus and detritus on the glass left by the anemone as it crawled during the attack.

TABLE IV

Feeding experiments to determine whether *Aeolidia papillosa* shows a preference for specific body regions when attacking *Anthopleura elegantissima*. In Experiment A the anemones were not attached and were lying on their sides so an approaching nudibranch had one chance to encounter the oral area first, two chances for the column and one chance for the pedal disk. In Experiment B, the anemones were attached and a nudibranch would typically make contact with a tentacle first, but then it would touch the column even if it attacked the tentacles. The results were significant at less than 0.01% using chi square.

Experiment	A		B	
	Predicted (%)	Actual (%)	Predicted (%)	Actual (%)
Column	16.5 (50%)	4 (12%)	30.5 (50%)	19 (31%)
Tentacles	8.25 (25%)	18 (54%)	30.5 (50%)	42 (69%)
Pedal disk	8.25 (25%)	11 (33%)	0 ¹	0

¹ Anemones were attached so pedal disk was not available.

18 animals had disappeared, presumably washed away by slight swells (about 30 cm). Of the 15 animals remaining, 13 survived.

The results of a separate experiment also suggest the unsuitability of the intertidal for *A. papillosa* in the late spring and summer. On 2 May, 1976, 18 small specimens of *A. papillosa* (mean size 15 mm) were placed among marked clones in protected habitats under the canneries. The animals were placed among clones where there were cracks and algae to provide refuges from desiccation. Three days later, only one nudibranch could still be found in the area where it was placed. By the eighth day, no specimens of *A. papillosa* remained in the vicinity. During the late spring and summer this protected habitat, which is shaded after 1200 hr is apparently too stressful for *A. papillosa*.

The behavior of the nudibranch attack and anemone responses have been described previously (Russell, 1942; Waters, 1973; Harris, 1973; Edmunds *et al.*, 1976), but they will be reviewed because it is relevant to understanding the mechanism behind the bulging behavior described by Edmunds *et al.*, (1976). Figure 1 illustrates the sequence of attack. Initial contact is typically between the nudibranch's rhinophores and the anemone's tentacles (Fig. 1a). The tentacles and nudibranch both retract (Fig. 1b). *A. papillosa* then moves into attack with buccal mass extended, rhinophores retracted and cerata bristled. The oral tentacles touch the column causing it to bulge in the area touched (Figs. 1c, d). The bulging behavior consists of an exaggerated relaxation of the column wall where contact has been made. Waves of contraction run up and down the column and changes in height and shape are frequent during bulging. This behavior continues for up to 6 hr after a single contact with *A. papillosa* mucus. A result of this behavior is to raise the tentacles out of reach of the nudibranch (Fig. 1c). If the *A. papillosa* does reach the tentacles, before bulging is initiated, it may be lifted free of the substrate (Fig. 1d).

Experiments testing for a preferred site of feeding by *A. papillosa* on *A. elegantissima* showed that the tentacles are selected over the column (Table IV). Nudibranchs also select the pedal disc over the column when anemones are not

attached. This preference for the tentacles is even more obvious when the attack sequence is observed; as in Figure 1c, nudibranchs often finally fed on the column, because the anemone's behavioral responses prevented easy access to the tentacles.

The bulging behavior of *A. elegantissima* is elicited by mucus from *A. papillosa* and also from the aeolid *Hermisenda crassicornis* which occasionally attacks anemones (Table V). That mucus from *A. papillosa* caused bulging was first indicated during attempts to observe the alarm response in a tidepool containing several anemones. An *A. papillosa* was placed in the pool to determine if feeding on one anemone would cause the alarm response in other individuals. The nudibranch crawled between two anemones, but did not attack either. Within minutes the contacted anemones had elongated their columns; they remained in this expanded and distinctive posture for over 2 hr. This indicated to us that mucus from the nudibranch may be initiating this behavior. Subsequent experiments (Table V) verified this hypothesis. Mucus from *A. papillosa* caused the column to bulge, and this behavior pattern lasted for 3 to 6 or more hours even without further stimuli from the nudibranch. In addition to inflation of the column on the side touched, the verrucae dropped their attached sand grains and shell bits.

Tests conducted to determine sites of sensitivity in *A. elegantissima* to *A. papillosa* mucus revealed that only the column was responsive. The tentacles contracted when touched by mucus, but did not elicit any inflation behavior by any part of the column and returned to their normal relaxed position within a minute or two. Howe and Sheikh (1975) showed that the tentacles were the site of greatest sensitivity to the alarm pheromone anthopleurine. Mucus from any part of *A. papillosa* and from *Hermisenda crassicornis* elicited column bulging, while mucus from *Anisodoris nobilis* had no observable effect on anemone behavior. Some anemones continued to show bulging reactions for 6 or more hours after a single contact with mucus from *A. papillosa* even in running sea water. Since 6 hr is the approximate time of submergence during a tidal cycle and *A. papillosa* only feeds during high tide, these results suggest a two-part mechanism in which anthopleurine causes a quick and short-term contraction of the tentacles which increases the likelihood that *A. papillosa* will contact the column first. Contact with the column will result in mucus from the nudibranch being

TABLE V

Summary of two experiments to test the effects of *Aeolidia papillosa* mucus on column bulging in *Anthopleura elegantissima*. Mucus secreted from glands on the foot and on the cerata was compared. Mucus from two other nudibranchs was also used; *Hermisenda crassicornis* occasionally attacks anemones, while *Anisodoris nobilis* eats only sponges and served as a control.

Source of Mucus	% Anemones bulging over time in minutes						
	0	min 60	120	180	240	300	360
<i>A. papillosa</i> foot (34) ¹	0%	76	62	53	29	18	6
<i>A. papillosa</i> cerata (37)	0%	81	84	81	62	32	22
<i>H. crassicornis</i> (37)	0%	65	84	78	51	24	22
<i>A. nobilis</i> (40)	0%	2.5	5	7.5	7.5	2.5	0

¹ Total number of anemones used in two replicate experiments.

sensed by the column receptors; this will initiate a slow but long-lasting inflation of the column that will raise the tentacles far above the substrate, often out of reach of the nudibranch.

Verification that *A. papillosa* mucus elicits bulging under field conditions occurred during desiccation experiment 3 (Table III). Nudibranchs were placed in clear areas within scattered clones. Water and mucus from the nudibranchs drained down the rock surfaces and around anemones below the nudibranchs. Thirty minutes after the experiment began, anemones which contacted the fluid draining from *A. papillosa* were bulging; many dropped their sand grain cover and several had released from the rock. The unattached anemones were lying on their sides with the column bulged, but the slope was too gentle for them to fall or roll from their original position without water movement. The next day the anemones were reattached, contracted and recovered with sand grains. However, comparison of photographs taken during the experiment and the following day showed that a number of anemones had moved 2 to 6 cm and four anemones had disappeared.

When attacked by *A. papillosa*, individuals of *A. elegantissima* crawl in the opposite direction. In 3 hr animals may have crawled as far as 4 cm. In the next 24 hr animals continued to crawl for another 3 to 4 cm, although limited observations suggest that in the field they may not crawl as far as they do in the laboratory.

In the laboratory, approximately 10% of the anemones attacked by *A. papillosa* released from the substrate during the attack. We do not know whether the percentage of release is as high in the field where there is active water movement, though both authors have encountered *A. papillosa* in tide pools in the process of attacking unattached anemones. Verrucae of detached anemones are extremely adhesive and attach quickly to any object that they contact. Unless there is active water movement at the time anemone releases, it may ultimately reattach in the same area.

DISCUSSION

The clonal form of *Anthopleura elegantissima* occurs primarily in the midtide zone on the exposed coastline of the west coast of North America (Hand, 1955; Ricketts and Calvin, 1962; Dayton, 1971; Francis, 1973a, b; Sebens, 1977). In this habitat individuals are exposed to wave action and six or more hours of exposure to air twice every 24 hr. Connell (1972) has suggested that the upper limit of a species in the intertidal is due to physical factors, especially physiological tolerance to exposure, and that the lower limits are due primarily to biological factors such as competition and predation. Dayton (1971) showed that *A. elegantissima* cannot survive in more protected areas of the San Juan Islands, because it is incapable of withstanding the long mid-day low tides in the summer. The exposure experiments (Table III) and collecting data suggest that specimens of *A. papillosa* are less able to withstand desiccation than their prey and that affects their ability to hold on to the substrate when the tide returns. The effect of desiccation appears to be greatest in the warmer months of the year and in protected areas such as Monterey Bay, since *A. papillosa* can be collected with *A. elegantissima* in the summer at exposed, open coastal sites (Waters, 1973).

A. papillosa presumably survives better in exposed habitats because spray from waves decreases the threat of desiccation.

The majority of large, reproductive *A. papillosa* found associated with clones of *A. elegantissima* have been at the lower end of the anemone's range in the intertidal. The mean size of *A. elegantissima* is greatest in these habitats (Sebens, 1977). Sebens (1977) has suggested that the longer submersion time increases feeding time for the anemones. A similar phenomenon has been described for the gastropod *Tegula funebris* (Paine, 1969). The largest specimens of *T. funebris* were found in the lower intertidal where the increased food supply and time for feeding would be translated into greater reproductive output. However, this was offset by increased threat of predation by the starfish *Pisaster ochraceus*. It is likely that the intertidal distribution of *A. elegantissima* represents at least a partial or seasonal refuge from predation by *A. papillosa* and that the lower limit of the anemone's range is influenced by where the balance between the potential for increased reproductive output and the threat of predation comes out in favor of predation.

Clone formation in *A. elegantissima* has been described as a competitive strategy that allows for rapid space utilization (Sebens, 1977). The fact that *A. elegantissima* has a well developed aggressive response to encroachment by other anemones similar to that reported for corals by Lang (1973) reinforces the likelihood that clone formation is an adaptation for space competition (Francis, 1975b, 1976; Purcell, 1977). Clone formation may also have adaptive significance as a defense against predation. Veligers of *A. papillosa* settle on the rock surface adjacent to their anemone prey (Harris, unpublished observations); therefore, only the periphery of a clone will be available for recruitment of the predator. The circumference of a clone of anemones is less than the sum of the circumferences of the same number of individual anemones were they dispersed, so there is less surface area for settlement. Also, the anemones at the center of the clone will be relatively free from predation (Table II) while those at the periphery of the clone will be exposed to the greatest damage from nudibranch attacks.

Francis (1976) has shown that anemones at the periphery of a clone serve as soliders which expend most of their energy in the production of nematocysts and regenerating wounded areas after aggressive encounters. These soldiers do not contribute directly to asexual or sexual reproductive efforts of the clone. Assuming that clone formation is primarily a competitive strategy similar to encrusting colonial growth as proposed by Jackson (1977), the additional adaptive value of minimizing the threat of predation for a majority of clone-mates should have a synergistic effect in reinforcing selection for cloning.

A. papillosa shows a clear preference for the tentacles of *A. elegantissima* (Table IV). The final feeding site is dependent on several factors including relative sizes of the predator and prey, the initial position of the tentacles, and the reaction of the anemone. If the length of the nudibranch is equal to or greater than the column diameter of the anemone, then the aeolid is large enough to readily attack the tentacles of an anemone in the normal open position (see Fig. 1a). Should the tentacles be raised in an alarm response or chance behavior, then the likelihood of the nudibranch reaching the tentacles is reduced, particularly if the anemone responds to contact with the column by bulging (Figs. 1c, d).

A. papillosa that have previously eaten *A. elegantissima* leak anthopleurine for up to 7 days and anthopleurine leaked from a nudibranch will initiate the alarm response (Howe and Harris, 1978). The alarm response, which involves a quick general contraction of the tentacles, may be of some advantage to an anemone in that it does increase the possibility that *A. papillosa* will not contact the tentacles first. A number of encounters have been observed in which the nudibranch gave up when it failed to reach the tentacles of an anemone. A specimen of *A. papillosa* feeds daily and primarily at high tide. The response to anthopleurine leaking from a nearby nudibranch should be greatest at the beginning of a high tide, since the anemones will have been free of the water-carried signal during low tide; continued exposure to anthopleurine causes fatiguing of the response over time (Howe and Sheikh, 1975).

The alarm response is of short duration and therefore not likely to deter a large, persistent nudibranch. However, raising the tentacles does increase the likelihood that the predator will touch the column. The column responds to *A. papillosa* mucus by swelling and this behavior pattern lasts for several hours (Table V).

There are two possible advantages to bulging behavior. The first is protection of the tentacles. It should be selectively advantageous to keep the feeding structures intact and instead to lose tissue from the column. Attacks to the column typically involve removal of epidermal tissue which will be regenerated, and very seldom result in complete penetration of the body wall. The second advantage is that when a specimen of *A. papillosa* attempts to reach or succeeds in reaching the tentacles, it must release at least partially from the rock surface (Figs. 1c, d) and will be vulnerable to being dislodged by wave action. Being washed off the rock by surge may not kill the nudibranch, but it removes it from further predation on the same clone.

The site of reception for the water-transmitted pheromone, anthopleurine, is the tentacles of *A. elegantissima*, and the alarm response involves short duration, generalized contraction of the tentacles (Howe and Sheikh, 1975). *Aeolidia papillosa* mucus is detected by contact, the receptors are in the column and the response is a localized inflation of the column. It is predictable that the receptors for a water-transmitted pheromone would be in the tentacles, for they extend farthest from the central axis of the animal. The generalized alarm response should be most effective if it occurs before contact is made with the predator since no directionality of response is required. It also seems likely that the receptors for a localized response to a slow-moving predator would be at the site of the response as is the case with the receptors for the bulging behavior.

Crawling by an anemone after an attack will potentially decrease the likelihood of a second attack because of the presence of the other members of the clone. Nudibranchs attack the first individual they encounter when foraging. This would spread the damage produced by a nudibranch to several members of a clone and decrease the chances for the loss of an individual and shrinkage of the clone. Another effect of crawling behavior would be to isolate the nudibranch on the rock substrate, increasing the chances of desiccation. This is most likely to occur in young nudibranchs that do not show a strong tendency to hide at low tide.

Approximately 10% of the encounters observed in the laboratory between *A. papillosa* and *A. elegantissima* resulted in the anemone releasing from the substrate. Rosin (1969) reported a similar escape response in *Anthopleura nigrescens* (Verrill) to its predator, the aeolid nudibranch *Herziella* sp. In the field, detaching from the rock substrate will potentially result in the anemone being carried to new habitats which may be viewed as a means of dispersal of the clone, and therefore, a positive side result of this association.

A specimen of *A. papillosa* consumes 50 to 100% of its wet body weight each time it feeds, which is at least once a day (Howe and Harris, 1978). Therefore, the nudibranch becomes an increasing threat as it grows to a point where it is capable of killing an anemone in a single meal; this suggests that the selective value of the defensive mechanisms discussed would be most effective against young nudibranchs. *A. papillosa* is about 0.5 mm in length when it first metamorphoses. Nudibranchs about 1 mm in length grow to about 35 mm and become sexually mature in a little over two months in the laboratory (Harris, in preparation). This suggests that the time and size must be considered in evaluating the selective value of defensive mechanisms since it will be at least 2 months after metamorphosis before a nudibranch is able to cause serious damage during an encounter or before it begins to reproduce.

None of the defensive adaptations described in this paper stop *A. papillosa* veligers from metamorphosing in a clone, nor are they effective in deterring predation in any given encounter between a nudibranch and an anemone either in the field or in the laboratory. We suggest that these mechanisms interact in such a way that the predator is killed before it reaches sexual maturity. The overall defensive strategy seems to be to minimize damage to clone members and to increase the likelihood that the predator will be killed or removed by desiccation and/or wave action.

The best evidence for this proposed defensive strategy is the fact that *A. papillosa* is primarily associated with the subtidal anemone *Metridium senile* (Harris, 1973, 1976, and in preparation). A similar pattern occurs in the Atlantic where *A. papillosa* is associated with *Metridium senile* (Stehouwer, 1952), *Sagartia elegans* (C. Todd, University of North Wales, personal communication) and *Cercus pedunculatus* (J. Tardy, University of Poitiers, personal communication); these three anemones were found to be among the least preferred in laboratory studies by Edmunds *et al.* (1975). Swennen (1961) reported large numbers of specimens of *A. papillosa* feeding on *Actinia equina*, a preferred species found in a similar habitat to that of *Anthopleura elegantissima*. Swennen's observations were made during the winter when the threat of desiccation would be least.

To influence the prey preference hierarchy of *A. papillosa*, the defensive strategies of anemones should focus on preventing the nudibranch from reaching sexual maturity and reproducing. Young nudibranchs seldom leave the prey where they have metamorphosed unless the prey is consumed or the nudibranch attains sexual maturity and searches for a mate (Harris, 1973); therefore, the choice of initial prey species takes place at the veliger stage prior to metamorphosis. The primary criterion for the choice of prey species must be survival to sexual maturity and reproduction. The prey preferences reported in the literature (Waters, 1973; Harris, 1973; Edmunds, *et al.*, 1975) are derived from laboratory

choice experiments with adult nudibranchs. Harris (1976) proposed that the prey preference hierarchy for the veliger stage of *A. papillosa* should be limited to relatively few species of anemone in an area and the principle criterion for selection should be survival to reproduction. In contrast, adult nudibranchs should be much less selective in their choice of prey since continued survival and reproduction should be the primary consideration at this stage. *A. papillosa* attains sexual maturity 2 to 3 months after metamorphosis and at a length of about 35 mm; an individual nudibranch is capable of continuing to reproduce for 6 or more months while growing to about 120 mm (Swennen, 1961; Harris, 1973; Clark, 1975). Adult specimens of *A. papillosa* show ingestive conditioning to even non-preferred anemones and will seek out prey they are conditioned to, unless they make contact with a more preferred species, and then they will switch (Harris, 1973; Wood, 1968; Murdoch, 1969). A hungry specimen of *A. papillosa* will attempt to feed on virtually any anemone species and even the corallamorpharian *Corynactis californica* Carlgren, 1936 (Waters, 1973; Harris, in preparation; Edmunds *et al.*, 1975).

In conclusion, the clonal form of *Anthopleura elegantissima* has evolved a series of defensive mechanisms including intertidal position, cloning, the alarm response to the pheromone, anthopleurine, column bulging initiated by nudibranch mucus, crawling and releasing from the substrate. None of these adaptations prevent veligers of *A. papillosa* from metamorphosing in association with *A. elegantissima* nor do they prevent an attack. However, they do combine in the context of the natural environment to form a very effective defensive strategy which increases the chances that the predator will be removed and/or killed by desiccation and/or wave action before it grows to sexual maturity. By preventing reproduction in the majority of nudibranchs which do metamorphose on this species, *A. elegantissima* exerts negative selective pressure on the prey preference hierarchy of the veliger stage. The success of this defensive strategy is illustrated by the fact that *A. papillosa* is primarily associated with one of the least preferred anemones of adult *A. papillosa*.

Mechanisms like cloning and the release of anthopleurine may also serve other functions such as competition or communication between clonemates. The fact that these mechanisms have adaptive value for more than one aspect of the anemone's biology should increase the selection for these traits.

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SUMMARY

1. The defensive mechanisms shown by the west coast, intertidal sea anemone, *Anthopleura elegantissima*, in response to its nudibranch predator *Aeolidia*

papillosa are identified and evaluated in the context of the environment where *A. elegantissima* occurs. The defensive mechanisms include intertidal distribution, clone formation, alarm response, bulging of the column, crawling and releasing from the substrate.

2. *A. papillosa* are primarily located at the periphery of clones so that anemones in the interior of the clone have a refuge from predation. Assuming that cloning is an adaptation for space competition in *A. elegantissima*, then the additional advantage derived as a defensive mechanism should increase selection for clone formation.

3. *A. papillosa* was less able than *A. elegantissima* to withstand desiccation from exposure at low tide. This suggests that the intertidal distribution of *A. elegantissima* is a defensive adaptation which reduces the threat of predation by *A. papillosa* at least during the warmer months of the year.

4. The bulging of the column at the site of contact was found to be a localized response of several hours duration. The mucus of *A. papillosa* stimulated the response and the receptors were found to be situated in the column. Mucus from the coelenterate-eating aeolid nudibranch, *Hermisenda crassicornis*, also initiated the response while neither the mucus from the sponge eating dorid, *Anisodoris nobilis*, nor control swabs dipped in sea water caused bulging.

5. None of the defensive mechanism directly protects an anemone from attack by *A. papillosa*. The defensive mechanisms all interact to minimize damage to the clone until the predator is removed by desiccation and or wave action. This strategy is most effective during the 2- to 3-month period between when the veliger metamorphoses and when the nudibranch reaches sexual maturity.

6. This defensive strategy of killing the young nudibranch before it reproduces may negatively influence prey selection by the veliger stage. The evolution of the prey preference hierarchy of the veliger stage should be based on the criterion of survival to sexual maturity. Evidence for the effectiveness of this defensive strategy is that *Acolidia papillosa* is primarily associated with the subtidal anemone, *Metridium senile*, one of the least preferred prey of adult nudibranchs.

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REPRODUCTION AND SURVIVAL OF THE PILEWORM *NEREIS SUCCINEA* IN HIGHER SALTON SEA SALINITIES

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The Salton Sea is a large (200 square miles) closed salt lake in a below-sealevel depression in the Colorado Desert of southeastern California. The Sea was formed accidentally from 1905 to 1907 when flood waters of the Colorado River broke through poorly constructed headgates of an irrigation canal. Following repair of the break, the new Salton Sea evaporated to a low of 250 feet below sea level by 1925. Since then the Sea has gradually increased in volume and surface elevation because agricultural waste water inputs exceed the annual evaporation of about 6 feet. In 1977, surface elevation was -228 feet, highest since 1914, and salinities averaged about 37‰ in summer. Early history of the Salton Sea is given by MacDougal (1914), and later history by Walker (1961).

The original biota of the Salton Sea, freshwater organisms from the Colorado River and a few species native to desert springs, died out by the mid-1920's. Attempts to introduce sportfishes did not succeed until the early 1950's, with the establishment of the croaker (*Bairdiella icistia*), sargo (*Anisotremus davidsoni*), and orange-mouth corvina (*Cynoscion xanthulus*), all from the Gulf of California. Several successful introductions of invertebrates have also occurred. The Salton Sea is eutrophic and highly productive (Walker, 1961), but as there are no planktivorous fishes, most plankton die and are converted to benthic detritus (Arnal, 1961). *Nereis* (*Neanthes*) *succinea* Leuckart, a polychaete annelid introduced in 1930, is the most important benthic detritivore and the most important food source for the sportfishes. The quasi-marine ecosystem of the Salton Sea is discussed by Walker (1961) and Young (1970). Ecology and reproductive biology of *N. succinea* in the Salton Sea have been discussed by Carpelan and Linsley (1961a, b) and Walker (1961).

With the salinity of the Salton Sea approaching 40‰ in the early 1970's, much concern has been expressed over the future of the sportfishery, one of the most important in California (Hanson, 1972). Increasing development of local geothermal energy resources may result in spills of highly saline waste brines into the Sea, which may also affect the sportfishes or their food supply (Shinn, 1976). Reproduction by the three sportfishes is limited to salinities below 40‰ (Brocksen and Cole, 1972; Lasker, Tenaza, and Chamberlain, 1972; May, 1975, 1976). There is little published information on high salinity adaptations in polychaetes (Bayly, 1972; Oglesby, 1978), and only one published study on the effects of higher salinities on *N. succinea* in the Salton Sea. Hanson (1972) observed that pileworms survived 96-hr exposures to salinities as high as 67.5‰, but speculated

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that "reproduction of the pileworm would probably be adversely affected at lower salinities."

It is the purpose of the present study to establish upper salinity limits for reproduction and survival of *N. succinea* in Salton Sea waters, in order to assess the possibility that pileworm failure due to increased salinity from either evaporation or brine spills may adversely affect the sportfishery.

MATERIALS AND METHODS

Pileworms were collected from the northeastern Salton Sea, at Mecca Beach in the Salton Sea State Recreational Area. Worms were picked by hand from shallow sediments, particularly from barnacle shell rubble cemented with gypsum crystals. While a few heteronereids could be collected from the benthos in this fashion, it was necessary to collect by night-lighting in order to secure adequate numbers for experiments on fertilization and development.

In the laboratory, worms were maintained in glass tubes in finger bowls, covered to reduce evaporation. For most experiments worms were maintained at 21 to 23° C; for some experiments, worms were kept at 34° C, somewhat warmer than summer water temperatures in 1976-77, but not as warm as maximum water temperatures of 36° C recorded by Walker (1961).

Salton Sea water was collected from Mecca Beach. Higher salinities were made by evaporating 36‰ Salton Sea water at about 50° C until it reached a concentration of about 90‰. This was then mixed with 36‰ Salton Sea water to provide a full range of salinities. During the concentration process, calcium salts precipitated. Thus, in the final mixtures, calcium may have been somewhat undersaturated. For some experiments media were made up from Instant Ocean, an artificial salt mixture resembling ocean sea water in ionic ratios. All media were routinely filtered through activated charcoal in Whatman Grade 3 filter paper before use. Media were changed in the culture dishes daily. No antibiotics were used.

Laboratory fertilizations were carried out according to the procedures detailed in Costello, Davidson, Eggers, Fox, and Henley (1957) and Smith (1964). Water was changed daily, involving some loss of developmental stages by decanting. In some cultures, bacterial or protozoan contamination was present; however, this did not pose a severe problem experimentally. Since no supplemental food was supplied to the developing larvae, cultures died at a late trochophore or early 3-setiger stage.

Salinities in the field and during ordinary laboratory operations were measured with an American Optical total solids refractometer, calibrated against known solutions measured with a Hewlett-Packard vapor pressure osmometer and with a Buchler-Cotlove chloridometer. Chlorinities were converted to ‰ Salton Sea water using the ion ratios given in Carpelan (1958), Walker (1961), and Young (1970).

RESULTS

Effects of higher Salton Sea salinities on survival of atokous worms

Experiments on survival of large atokes (immature worms) involved either direct transfers to the full range of salinities, or gradual increases of salinity in

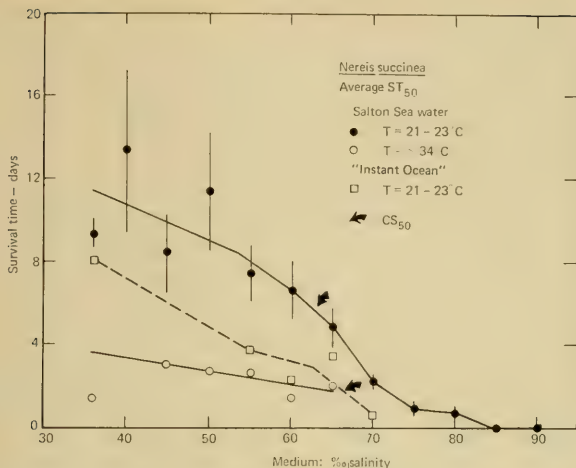


FIGURE 1. Survival time of *Nereis succinea* in increasing concentrations of Salton Sea water, expressed as average ST₅₀. Points are averages of all trays (initially 10 worms each) in each salinity, with the standard error indicated. Arrows indicate the Critical Salinity (CS₅₀) where survival is reduced to 50% of that in 36‰ Salton Sea water.

increments of 10‰ until the final test salinity was reached. Calculation of the time for 50% survival (ST₅₀) of a test group of worms (usually four trays with 10 worms each) began with the day the worms first were placed in the final test salinity. Graphs of ST₅₀ as a function of the final salinity were used to determine the Critical Salinity (CS₅₀), the salinity where survival is reduced to 50% of the survival in 36‰ Salton Sea water. This calculation of CS₅₀ is possibly biased in those experiments involving graduated salinity increases, since it ignores the mortality of worms in lower salinities during the adaptation period. However, otherwise comparable experiments in which worms were transferred directly to the final salinities gave similar results. Results of both types of experiments are combined for the following analyses.

Results for all experiments on survival of atokes are summarized in Figures 1 and 2. These are averages and calculations based on over 280 worms in 36‰, over 140 worms in each salinity between 45 and 60‰, and between 50 and 100 worms in all higher salinities. Figure 1 shows the average survival time (ST₅₀) for all worms in all salinities, with the standard error indicated. Survival was high at salinities of 50‰ and lower, but declined in higher salinities. The critical salinity CS₅₀ is estimated to be slightly lower than 65‰. Figure 1 also provides the results of two related experiments: the effects of elevated temperature (34°C rather than the usual 21–23°C), and the effects of transfer to Instant Ocean. In both cases, overall survival seems to be diminished as compared with worms in

Salton Sea water in 21 to 23° C, though the general pattern is similar. For worms in Instant Ocean, CS_{50} is estimated to be somewhat higher than 65‰. It was not possible to provide an estimate of CS_{50} for the worms at 34° C, for it was higher than the highest salinity used in this experiment, 65‰. It appears that the Critical Salinity is not lowered for Salton Sea pileworms exposed either to ocean sea water or to temperatures only slightly lower than maximum summer water temperatures in the Salton Sea.

Much of the variability in the estimates of average ST_{50} (Fig. 1) is caused by the fact that in all salinities, all the worms in a given tray often would die soon after the first worm died, presumably because of the accumulation of toxic materials before the medium was changed. Not only is variability increased by this phenomenon, which may not be salinity-related, but also the average ST_{50} is decreased. Die-off of entire trays of 10 worms was particularly a problem at 34° C, due to increased bacterial activity. In order to provide an estimate of survival that avoids this problem, Figure 2 shows the survival time for the longest surviving worm in each salinity. At least some pileworms survived longer than the length of the experiments in nearly all salinities of 65‰ and lower, over 4 weeks for the worms in Salton Sea water at 21 to 23° C, and over 2.5 weeks for the worms in Instant Ocean and in 34° C. At higher salinities maximum survival time declined rapidly. No worms survived even a day at salinities higher than 85‰ at 21 to 23° C, nor higher than 75‰ in Instant Ocean. In both these experiments, CS_{50} was 70‰. No salinities higher than 65‰ were used in the experiments at 34° C, and since at least one individual lived as long as 8 days in this salinity, it is not possible to estimate CS_{50} , which must be higher than 65‰.

Most worms in the laboratory did not survive more than a week or two, even when maintained in 36‰ Salton Sea water. Carpelan and Linsley (1961a) indicated that *N. succinea* takes over a month to reach sexual maturity in the Salton Sea, so presumably pileworms must live well over a month in their natural habitat waters. Diminished survival in the laboratory may be a consequence of accumulation of metabolites, to lack of adequate food, or to other artificial causes. For these reasons, the survival times of pileworms in these laboratory experiments should not be taken as an exact counterpart of survival in the Salton Sea as salinity there increases. Rather, these laboratory results should be taken as an index of survival.

Both indices of survival (Figs. 1, 2) indicate that atokous pileworms survive increased Salton Sea salinities up to 60 to 65‰. Survival is considerably diminished at salinities in excess of 70‰, with no survival above 85‰, a concentration which is well over twice the present concentration of the Salton Sea. Even high summer water temperatures of 34° C do not decrease the survival limits of the pileworms. There is no indication that pileworms live longer in ocean water than in Salton Sea water.

Effects of higher Salton Sea salinities on heteronereid development

Studies of sexual development and spawning indicate that while *N. succinea* breeds year-round in the Salton Sea, reproductive activity is somewhat depressed during the summer months of high water temperature (Carpelan and Linsley,

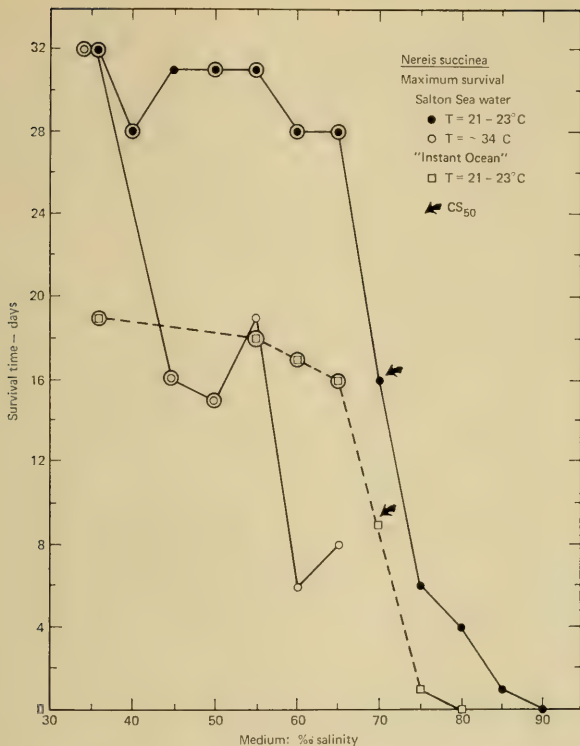


FIGURE 2. Maximum survival time of *Nereis succinea* in increasing concentrations of Salton Sea water. Circled points are those where worms were still alive by the end of the experiment.

1961a, b). Nevertheless, heteronereids (sexually mature adult worms) were collected throughout the summers of 1976 and 1977. The sex ratio of worms collected from the benthos at Mecca Beach was about one-third males and two-thirds females. As noted by Costello, *et al.* (1957) and Carpelan and Linsley (1961a), the sex ratio of swarming heteronereids is strongly reversed, males outnumbering females by about 10 to 1. This was also the case in our own collections. Heteronereids regularly developed in laboratory cultures, with the sex ratio comparable to that encountered in benthic worms during collection, about 2:1 females to males.

Heteronereid development in the laboratory was strongly influenced by salinity

(Fig. 3). About 15% of the pileworms maintained in low salinities (36 and 40‰) matured as heteronereids during the summer of 1977. A somewhat lower percentage (just under 12%) matured in 50‰, but in higher salinities maturation of heteronereids was greatly depressed, and there was no maturation in salinities of 70‰ or higher. There may be a differential effect of higher salinities on maturation of the two sexes, development of males being more strongly depressed than that of females. This observation may be an artifact of very small numbers of hetero-

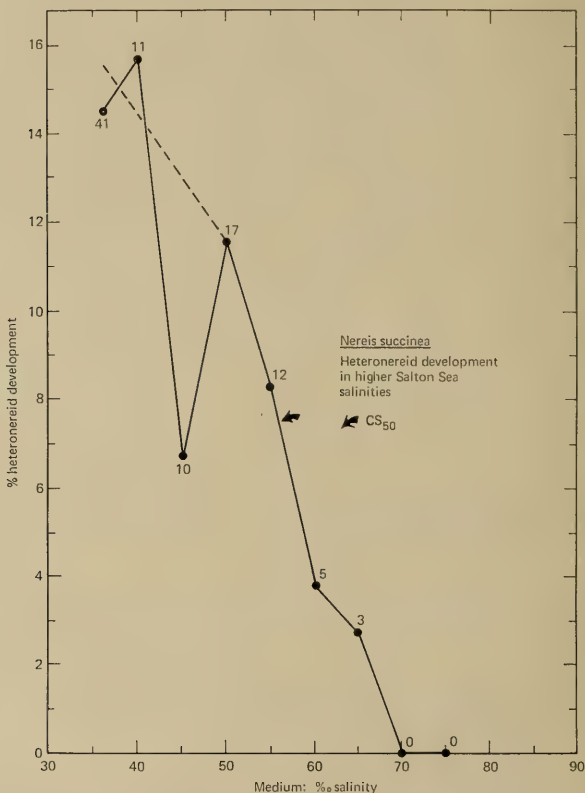


FIGURE 3. Effect of increasing Salton Sea salinities on heteronereid production by *Nereis succinea*. Actual numbers of heteronereids produced are indicated. Arrow indicates CS₅₀ where heteronereid production is reduced to 50% of that in 36‰ Salton Sea water.

nerereids in the higher salinities. The estimated CS_{50} for heteronereid development is about 55‰ (Fig. 3).

Effects of higher Salton Sea salinities on fertilization and larval development

Laboratory fertilizations, using heteronereids either collected directly from the Salton Sea or produced in laboratory cultures, were readily accomplished. Development of larvae in 36‰ Salton Sea water appeared normal, and had the same time course as in previous studies on Salton Sea worms (Carpelan and Linsley, 1961a, b) and on this species at Woods Hole (Costello, *et al.*, 1957). In all these studies, temperatures were similar, about 20 to 25° C.

Fertilization in various salinities. Fertilization of eggs of *N. succinea* could be accomplished at salinities as high as 50‰, although the percentage of successful fertilizations was much reduced at 50‰. In one experiment, there was less than 5% successful fertilization at 55‰, and in a second experiment, no success at this salinity. In these experiments, eggs from females maintained in 36‰ were initially transferred to culture dishes containing 100 ml of the experimental salinity, and sperm from males opened in 36‰ added. Eggs transferred to salinities of 45‰ and higher shrank from osmotic water loss immediately after the transfer. Shrinkage was particularly noticeable in 50‰ and higher, many eggs becoming greatly distorted. It is estimated that the CS_{50} for fertilization is 45‰. While there was a small proportion of apparently successful fertilizations in 55‰ in one experiment, there was no further development even to the first cleavage. The highest salinity in which any development takes place following fertilization is 50‰. These results are summarized in Figure 4.

Fertilization in 36‰ Salton Sea water, followed by transfers to various salinities. Smith (1964) demonstrated that later developmental stages of *N. diversicolor* were less sensitive both to lower and to higher salinities than early stages. To determine if this is the case with *N. succinea*, several experiments were conducted in which eggs were fertilized in 36‰ Salton Sea water and embryos transferred to higher salinities at several different times during development.

In the first group of experiments, eggs were transferred as soon as possible after fertilization into a full range of salinities. There was excellent survival and subsequent development in salinities up through 45‰, although in 45‰ there was some evidence of shrinkage, and a few zygotes cleaved abnormally. These abnormally cleaving embryos (such as "dumbbells" which cleaved no further, and irregular cell clusters) did not develop successfully. There was poor survival of embryos transferred to 50‰, with abnormal cleavages and much shrinkage, but a few normal trochophores developed at this salinity. At 55 and 60‰ there was temporary survival, a high proportion of abnormal cleavage patterns, and no successful development. Embryos transferred to salinities higher than 60‰ shrank excessively and showed no type of cleavage. These early cleavage stages seem as sensitive to higher salinities as fertilization itself; it is estimated that the CS_{50} for early cleavage is 45‰, with no successful development higher than 50‰. These results are summarized in Figure 4.

This same experiment was conducted at 34° C to determine if summer water temperatures were more stressful on development than 21 to 23° C. There were

no observed differences in survival and developmental success between 34° C and 21 to 23° C, other than a considerable acceleration of cleavage at the higher temperature. There seems to be no additional stress on development in temperatures as high as summer water temperatures in the Salton Sa. On the other hand, in either temperature regime, development in salinities of 45‰ was markedly delayed as compared with development at lower salinities.

In a second group of experiments, fertilized eggs were allowed to develop in 36‰ Salton Sea water until the early trochophore stage, about 8 to 10 hr. These early trochophore stages were then transferred to salinities up to 75‰. There was excellent survival and development at all salinities up through 50‰. No shrinkage of trochophores was noticed at 45‰. In 50‰ there was some shrinkage, and trochophore cilia ceased beating temporarily. At 55‰ these shock effects were more severe, but there was considerable survival and subsequent development. At 60‰ there was no recovery of ciliary motion following transfer, and while it was estimated that perhaps 5% of the early trochophores survived the transfer, there was no subsequent development. There was no survival at higher salinities. Thus, early trochophores are less sensitive to high salinities than earlier cleavage stages. The estimated CS_{50} is 50‰, but there was no development above 55‰. These results are summarized in Figure 4.

In the final experiment, fertilized eggs were allowed to develop in 36‰ Salton Sea water until the swimming trochophore stage, about 24 hr. Swimming trochophores were then transferred to salinities up to 75‰, and survival and subsequent development monitored to the beginning of segmentation about 18 hr later. Survival was not as good as in the experiments involving transfers at earlier stages, but there was some survival and development up through 55‰. Swimming trochophores transferred to 50 and 55‰ showed some shrinkage, and ciliary activity temporarily stopped. In 60‰ these shock effects were more severe, and survival was low; however, some trochophores were still alive after 18 hours, though development had not progressed. In higher salinities there was little or no survival following transfer, shock effects were very severe, and there was no subsequent development. It appears that swimming trochophores have about the same sensitivity to higher Salton Sea salinities as do early trochophores. These results are summarized in Figure 4.

DISCUSSION

The present experiments supplement and extend the results to Hanson (1972) in showing that atokous *N. succinea* can survive for extended periods of time in very high Salton Sea salinities, at least as high as 65‰, with perhaps some reduction of survival in 70‰. There is short term survival in salinities as high as 80‰, more than twice the present salinity of the Salton Sea. There are reports of nereid and other polychaetes in high salinities in lagoons and estuaries, but few reports are for salinities in excess of 50‰ (Bayly, 1972; Oglesby, 1978). There have been no experiments on any of these other polychaetes to determine actual upper salinity tolerances, or if they actually breed in such high salinities.

Hanson's (1972) prediction that reproduction of the pileworm in the Salton Sea would be adversely affected at salinities lower than the limit for adult sur-

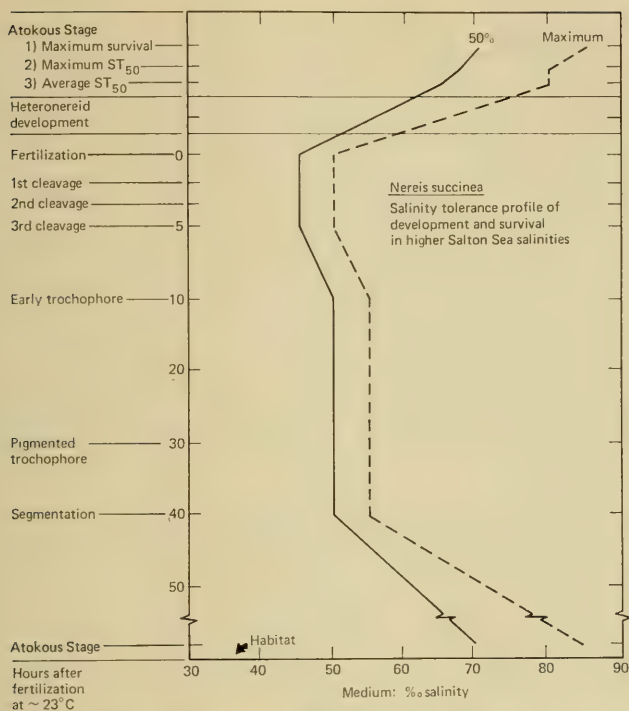


FIGURE 4. Salinity tolerance profile of development and survival of *N. succinea* in higher Salton Sea salinities. Stages and times are approximate for 21 to 23° C. The solid line indicates the Critical Salinity (CS₅₀), where survival and development are reduced to 50% of that in 36‰ Salton Sea water. The dashed line indicates the maximum salinity at which any survival or development occurred.

vival is confirmed by these experiments, for there was no successful fertilization or cleavage in salinities higher than 50‰, and reasonable success was limited to salinities of 45‰ and lower. Early and late trochophores could tolerate somewhat higher salinities, up to 50‰. Assumption of the high salinity tolerance of the atokes must come later in development than the appearance of segmentation, the termination of the present experiments.

Table I summarizes the literature on the effects of low salinities on development of *N. succinea*. Taken together, these reports suggest that *N. succinea*, like many other estuarine and marine polychaetes, cannot reproduce at salinities below the horohalinicum of 5 to 8‰ (Oglesby, 1978), even though atokous *N. succinea* can

survive at considerably lower salinities (Oglesby, 1965; Hogue and Oglesby, unpublished results). The report by Foster (1972) that *N. succinea* can survive in fresh water is not supported by published data (Oglesby, 1965, 1978). The studies summarized in Table I, combined with the present results, provide a picture that is comparable to *N. diversicolor* (Smith, 1964), showing a "bottleneck" of salinities above and below which cleavage is blocked (Fig. 4). That is, while adult and atoke survival limits range from about 1‰ to as high as 80‰, development is successful only between 10 and 45‰.

It is premature to conclude that when the Salton Sea finally exceeds 45 to 50‰, reproduction of *N. succinea* will be blocked. As Smith (1964, 1977) has discussed, at least some of the reason for lack of reproductive success at extreme salinities may be mechanical, due to osmotic swelling in low salinities and osmotic shrinkage in high salinities. Shrinkage was very apparent in the present experiments at 50‰ and higher in eggs and embryos transferred from 36‰. It would have been desirable to attempt fertilizations with eggs and sperm taken from adults adapted to much higher salinities, to avoid the initial osmotic problem. However, even though heteronereids were produced in the laboratory in salinities as high as 65‰ (Fig. 3), never were a male and a female mature at the same time in a salinity higher than 36‰. Smith (1964) reported that some populations of *N. diversicolor* in northern Europe reproduce in salinities greater than the upper salinity limit for reproduction of other populations of the same species. It may

TABLE I
*Effects of lower salinities on development of Nereis succinea**

Stage and treatment	Observed effects on development	Reference
Unfertilized eggs	Survival down to 9.6‰.	Just (1928)
Fertilized eggs	Very low resistance to dilute sea water.	Just (1928)
Development in 91‰ SW after 1 hr exposure to dilute SW	Normal development above 16‰; diminished success at 11–14‰, though some eggs develop normally.	Just (1930a)
Development in varied SW after fertilization in 91‰ SW	Normal development above 16‰; diminished success at 14‰, but some normal development; cleavage but not trochophores in 11, 13‰.	Just (1930b)
Development in varied SW after fertilization in 100‰ SW	Normal development of nectochaeta larva in 14–35‰; normal development of trochophore in 8‰; no cleavage in 2–6‰.	Kinne (1954)
Development in varied SW and temperatures after fertilization at 20‰	Development at 10, 15, 20‰, except none at 10‰ at 10° C. Development accelerated at higher salinity and higher temperature.	Dean and Mazurkiewicz (1975)

* *Nereis limbata* Ehlers, the name used by Just (1928, 1930a, b) is synonymous with *N. succinea* Leuckart. Just (1928, 1930a, b) did not provide the actual concentration of his "100‰ SW." Salinities presented in this table are based on data of Cole (1940) for summer water salinities at Woods Hole. Since there are no significant fresh-water inflows in the area, it is reasonable to assume that Woods Hole summer sea water averages about 32‰, or 91‰ of 35‰ salinity.

be that as the salinity of the Salton Sea gradually rises, there will be genetic selection for *N. succinea* with higher limits for reproductive success.

Developmental events were somewhat slowed at higher salinities, but at salinities below 45 to 50‰ development was otherwise normal. Smith (1964) observed that low salinities also delayed development, particularly cleavage, in *N. diversicolor*.

The present experiments indicate that temperatures as high as 34° C do not have any marked effect on adult survival and reproduction in *N. succinea*. At the present time there is year-round reproduction in the Salton Sea, and this pattern should continue as long as the overall salinity itself does not become too high.

Interestingly, Salton Sea pileworms do not seem to have reduced survival in Salton Sea water as compared with ocean water (Figs. 1, 2). May (1976) found that eggs and larvae of the croaker *Bairdiella* survived well in sea water, but had very poor survival in Salton Sea water of the same salinity. He suggested that this poor survival of the fish was related to the unusual ionic composition of the Salton Sea. This seems not to be a problem with *N. succinea*.

Spills of geothermal waste brines of 5 to 10 times the salinity of the Salton Sea (California Department of Water Resources, 1970; Shinn, 1976) would be expected to eliminate pileworms from the affected area, as well as other benthic and pelagic organisms. These adverse effects could be caused not only by the excessively high salinity *per se*, but also by concomitant elevated temperatures or reduced oxygen concentrations. Pileworm larvae are in the Salton Sea plankton all year (Carpelan and Linsley, 1961a, b), and so there would be a constantly available source for recolonization. Only if there were contamination by heavy metals would there be a long-term problem (Reish and Carr, 1978). Thus, it is unlikely that a spill of even highly saline geothermal waste brines would have any more than a temporary and localized effect on the population of *N. succinea* in the Salton Sea.

The present experiments indicate that reproduction of *N. succinea* in the Salton Sea will continue with undiminished success at salinities at least as high as 45‰, and probably as high as 50‰. This means that gradually increasing salinities of the Salton Sea will not adversely affect the pileworm until some years after the collapse of the sportfishery, which seems sensitive to salinities no higher than 40‰.

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SUMMARY

The polychaete annelid *Nereis* (*Neanthes*) *succinea* is the major benthic detritivore in the Salton Sea, an inland salt lake in southeastern California, and is critical in the trophic chain leading to the sportfishery. In view of the increasing salinity of the Salton Sea, laboratory experiments were conducted to determine critical upper salinity limits for reproduction and survival of pileworms. Atokous (immature) pileworms can survive for extended periods in Salton Sea salinities at least as high as 65‰, with some reduction of survival in 70‰, and with only short term survival in 80‰, more than twice the present salinity of the Salton Sea (36‰). Heteronereid production is depressed by salinities higher than 50‰. Reproduction of *N. succinea* is successful at salinities at least as high as 45‰, and probably as high as 50‰. Fertilization and early cleavage stages are less tolerant of elevated salinities than are later development stages such as trochophores.

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BEHAVIORAL RESPONSES OF *BALANUS IMPROVISUS* NAUPLII TO LIGHT INTENSITY AND SPECTRUM

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Although barnacle larvae have been used in numerous classical studies on phototaxis and spectral response, results have been predominantly qualitative in nature. Groom and Loeb (1890), Ewald (1912), and Rose (1925) studied phototaxis in stage I-II nauplii of *Balanus perforatus*. Vischer and Luce (1928) attempted to define the spectral sensitivity of cyprid *Balanus amphitrite* and "*B. improvisus*". Barnes, Crisp, and Powell (1951) demonstrated orientation to light during settlement of cyprid *Semibalanus balanoides* and *Balanus crenatus*. Based on these and other studies, Thorson (1964) characterized barnacles as maintaining positive phototaxis throughout their larval life.

More recent studies have begun to quantify light responses of barnacle larvae. Barnes and Klepal (1972) determined the spectral sensitivity and threshold of photo-response at 522 nm for stage I nauplii of *Elminius modestus* and *S. balanoides*. Crisp and Ritz (1973) studied the effects of dark-adaptation on relative light sensitivity for stage II *E. modestus* and *S. balanoides*, and limiting intensities of white light for photo responsiveness of stage II *S. balanoides* and *B. crenatus* and cyprid *S. balanoides*.

With the exception of a series of studies on the zoea of the estuarine decapod crustaceans (Forward, 1974; Forward and Costlow, 1974; Forward, 1976b, 1977; Forward and Cronin, 1978), quantitative phototactic and spectral studies of larval crustacean groups are sparse (see Forward, 1976a; Aiken and Hailman, 1978).

In this study, short-term phototaxis (direction) and orthophotokinesis (velocity) of *Balanus improvisus* nauplii are investigated using a new method of video-computer quantification which greatly facilitates studies of movement in small organisms (Davenport, Culler, Greaves, Forward, and Hand, 1970). The ability to rapidly quantify and compare movement parameters makes this system a potentially powerful tool for behavioral bioassays (Anderson, 1971; Olla, 1974). Thus this study demonstrates the capabilities of this system and provides a rigorous analysis of the photobiology of barnacle larvae.

MATERIALS AND METHODS

Experimental animal

Balanus improvisus nauplii were sorted from surface plankton tows taken at Pettaquamscutt River near Narragansett, Rhode Island. During the collection period (24 Oct-28 Nov., 1977) *B. improvisus* nauplii were abundant and easily

¹ Duke University Marine Laboratory, Beaufort, North Carolina 28516; and Zoology Department, Duke University, Durham, North Carolina 27706.

sorted from plankton samples (Lang, 1979). Water temperature and salinity at collection times ranged from 7 to 14° C, 13 to 20‰. Nauplii were immediately transferred to filtered sea water at 15° C, 15‰ and placed in either 15° or 20° C temperature boxes with constant illumination. For experiments conducted at 30‰, the temperature of the 15‰ water was first allowed to equilibrate at 20° C and nauplii were then transferred to 5‰ salinity increments at one hr intervals.

All larvae were maintained overnight (8–12 hr) at specified temperature salinity before being used experimentally. *Isochrysis galbana* and *Tetraselmis suecia* were added as food. The following morning, nauplii of the desired stage were sorted from initial cultures and groups of 20 transferred to 5-ml beakers. Light-adapted nauplii were exposed to room lights supplemented with a 60-W incandescent bulb for at least 1 hr prior to experimentation; dark-adapted nauplii were kept in dark temperature-controlled boxes for at least 1 hr before experimentation. Studies with other arthropods indicate that these times are adequate for light and dark adaptations (e.g., Hamdorf and Schwemer, 1975; Barnes and Goldsmith, 1977). To minimize effects of possible larval diurnal cycles (Singarajah, Moyse, and Knight-Jones, 1967), all experiments were conducted between 13:00 and 16:30 hr. All nauplii were tested within 26 hr of capture.

Light stimulus and video system

A microscope and closed circuit television system were used to monitor and record swimming behavior of nauplii. Dark field substage illumination, interference filtered to 830 nm (about 15 nm half width), provided light for a Cohu 4400 television camera mounted on a Wild M-5 microscope body (Lang, Lawrence, and Miller, 1979). Larvae were placed in a 1.2 × 1.2 × 1.0 cm lucite cuvette filled to 0.5 cm depth. Movement was monitored in the horizontal plane.

A light stimulus presented horizontally and perpendicular to the cuvette wall was provided by two sources. For initial studies on light response of nauplii, a grating monochromator with 150-W xenon short arc lamp (Oriel Corporation) was used. For spectral and intensity studies a slide projector with 300-W tungsten bulb and thin film absorption filters (Ditric Optics) ranging from 440 to 640 nm in 20-nm intervals (about 7 nm half-width) was used (Latz and Forward, 1977). In both cases light intensity of quantum levels were regulated by neutral density filters. Intensity was measured by a YSI model 64A radiometer.

Experimental procedure

Experiments were conducted in a temperature-controlled, darkened room. A preparation of 20 nauplii was transferred from a 5-ml beaker to the test cuvette; the cuvette was then aligned on the dark field stage. Light-adapted nauplii were transferred under room lights; dark-adapted nauplii were transferred under dim light (interference filtered at 700 nm). All lights were extinguished and nauplii were allowed at least 30 sec prior to experimentation to recover from movement of the test cuvette during placement on stage.

For initial studies on naupliar response to light stimulus, five intensities of 480-nm light ranging from 27 to 0.0027 W m² were used. Replicate preparations of nauplii were tested at each intensity in ascending order with 45-sec intervals

between 2.5 sec stimuli. The response of nauplii 2.5 sec prior to stimulation, during stimulation, and 3.0 sec following stimulation were analyzed by computer.

For spectral response, the 830-nm substage light was turned on 5 to 15 sec prior to light stimulus, a 2.5-sec stimulus applied, and the substage light extinguished. After 1 minute, a second wavelength stimulus (in ascending order) was applied. After four exposures the preparation of dark-adapted nauplii was changed. For both spectral and intensity studies, computer analysis was limited to naupliar response during the latter 2.0 sec of the 2.5-sec stimulus.

The general procedure for testing response to light intensities was similar. One preparation of nauplii was exposed to a complete sequence of seven intensities of 480-nm light starting with an intensity estimated as subthreshold. All stimuli were 2.5 sec in duration with 30- to 40-sec intervals between each stimulus.

At least three replicates were run with each plankton sample. With the exception of stage III nauplii, at least two different plankton samples were represented.

Computer analysis

Video recordings of naupliar response to light stimuli were played back through a video-to-digital processor, the "Bugwatcher" (Greaves, 1975). Outlines of each nauplii within the camera field of view are delimited as X-Y coordinates. For this study, video tapes were analyzed by computer at 10 frames per second; every sixth frame of the normal 60 f/sec recording was fed into a Data General Eclipse S/200 computer. Time series of X-Y coordinates (video files) indicating displacement of nauplii at 0.1-sec intervals were generated for each replicate sample. A tone generator synchronized with the light stimulus shutter control marked periods of stimulation; a tone detector in the Bugwatcher determined light stimulus duration to the nearest 0.1 sec on video files.

Video files were analyzed using second generation programs developed by Wilson and Greaves (1979). Processed video files (see Greaves, 1975) yielded a time-scaled computer track of naupliar movement (Fig. 1A). About 10 to 15 nauplii were tracked simultaneously. Linear velocity (Fig. 1B) and direction of travel (Fig. 1C) were calculated for each 0.1-sec interval of the tracks; mean values for individual nauplii during a given time interval were determined (Fig. 1D). Mean direction of travel (DOT) and mean linear velocity (MLV) for all nauplii of an experimental group were then pooled to calculate sample means. Sample DOT distributions were tested using a Chi-square test (Batschelet, 1965). Standard SPSS (Nie, Hull, Jenkins, Steinbrenner, and Bent, 1975) and SAS (Bar, Goodnight, Sall, and Helwig, 1976) analysis of variance programs were used to test sample MLV data.

RESULTS

Initial studies on stage II naupliar light response

Preliminary observations indicated that stage II *B. improvisus* nauplii were strongly positively phototactic to lower light intensities (about 10 W/m²), particularly near 500 nm. The following experiments were devised to further characterize the responses of light-adapted nauplii.

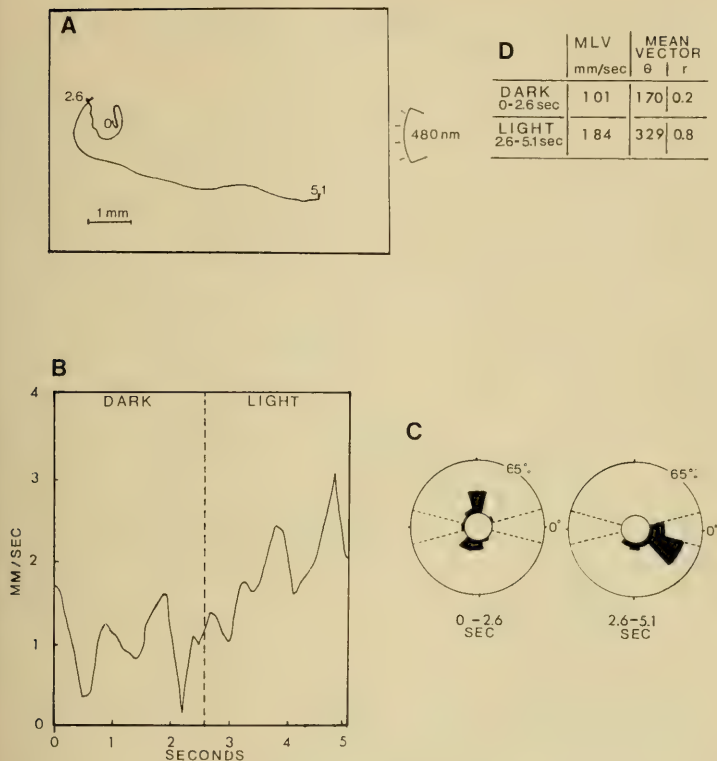


FIGURE 1. An example of computer analysis of a single *Balanus improvisus* nauplius. Digital processing of a video tape produces a computer tracking of the nauplius (A). A light stimulus (480 nm, 0.06 W/m^2) was applied from 2.6 to 5.1 sec. Linear velocity (B) and direction of travel (C) are calculated at 0.1-sec intervals (stimulus at 0°) and mean values for desired time intervals (D) determined.

Light-adapted stage II nauplii (15° C , 20‰) were exposed to 480 nm light at five intensities ranging from 27 to 0.0027 W/m^2 . The initial MLV of nauplii exposed only to 830-nm darkfield illumination does not vary significantly ($P = 0.05$) between sample group (Table I). During light stimulation, the MLV for groups at the upper three light intensities increases significantly; recovery to MLV statistically equal to control levels does not occur within the three sec following stimulation. No significant change in MLV occurs at the lower two light intensities

(Table I). Plotting the running mean velocities of each sample group at 0.1-sec intervals shows the time sequence of naupliar photokinetic response (Fig. 2). The change in MLV is delayed in onset and return to normal, relative to light stimulus duration.

A change in MLV (orthophotokinesis) is observed only above 0.027 W m⁻². However a directional response to light (phototaxis) occurs at all intensities (Table I, Fig. 3). DOT distributions during the initial "dark" interval do not vary significantly ($P = 0.05$) from a theoretical uniform distribution. During the "light" interval, nauplii at all light intensities exhibit nonrandom distributions and a significant difference from initial distributions. Computer plotted histograms (Fig. 3) illustrate a directed response toward the light source.

The individual nauplii paths (*e.g.* Fig. 1A) and time analysis of MLV responses (Fig. 2) indicate that a delay of about 0.5 sec often occurs between stimulation and naupliar response. The DOT distribution for the entire 2.5-sec light stimulus interval clearly indicates a general positive phototactic movement (Fig. 4A). If the initial 0.5-sec "orientation period" at light stimulation is omitted from DOT determinations, a strong directed response becomes evident (Fig. 4B). For the following experiments, mean naupliar direction and MLV during the latter two sec of a 2.5-sec light stimulus are presented. Positive phototaxis is considered a mean direction of travel $\pm 15^\circ$ of the light source; negative phototaxis is a DOT $\neq 15^\circ$ in the opposite direction. Comparative results using a $\pm 45^\circ$ "windows" in respective directions are also included.

To correlate with other laboratory studies (Lang, *et al.*, 1979; Lang, Miller, Lawrence, Marcy, and Clem, in progress), light intensity and spectral experiments were conducted at 20° C, using a tungsten light source and filters. Essentially the same positive phototaxis to 480-nm light was found using the new light source and at the higher temperature.

TABLE I

Mean linear velocity and χ^2 directional response comparison of light-adapted stage II *Balanus improvisus* nauplii (15° C 20‰) during initial "dark" interval (830-nm substage light) at 0-2.5 sec, 480-nm light stimulation at 2.5 to 5.0 sec, recovery "dark" interval at 5.0 to 8.0 sec. A null hypothesis that the distribution of direction of travel means (60° intervals) for each sample group of 30 to 38 nauplii is equal to a theoretical uniform distribution was tested using the χ^2 test. χ^2 values for $P \leq 0.05$ are indicated by an asterisk where P represents the probability of rejecting the null hypothesis when actually true.

Intensity W/m ² at 480 nm	Mean linear velocity mm/sec			Direction of travel Chi-square: experimental vs. uniform distribution		
	Initial "dark"	Light stimulation	Recovery "dark"	Initial "dark"	Light stimulation	Recovery "dark"
27	1.35 $\pm$ 0.35	2.13 $\pm$ 0.65	1.97 $\pm$ 0.63	7.14	33.67*	8.80
2.7	1.54 $\pm$ 0.55	2.29 $\pm$ 0.66	1.91 $\pm$ 0.61	4.43	64.14*	5.00
0.27	1.25 $\pm$ 0.39	1.82 $\pm$ 0.52	1.66 $\pm$ 0.55	7.86	29.81*	15.29*
0.027	1.46 $\pm$ 0.36	1.60 $\pm$ 0.45	1.55 $\pm$ 0.48	5.00	61.50*	8.74
0.027	1.46 $\pm$ 0.31	1.51 $\pm$ 0.38	1.50 $\pm$ 0.36	4.42	19.60*	8.47

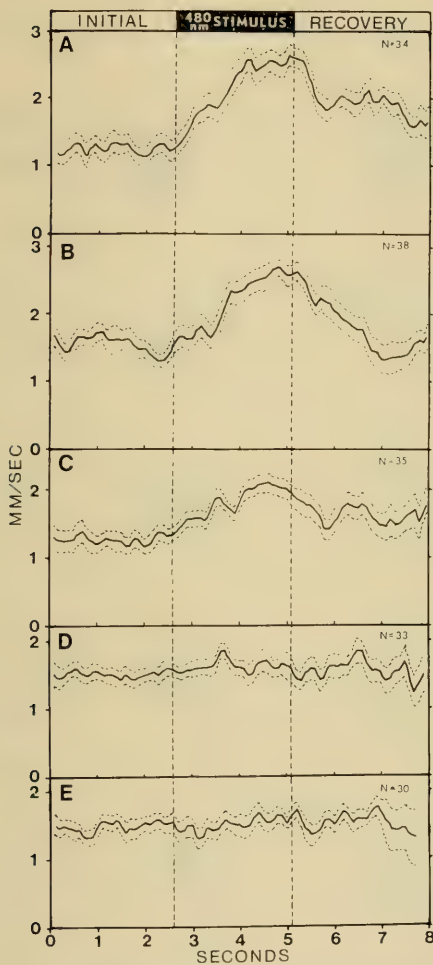


FIGURE 2. The running mean linear velocity $\pm$ variance of stage II *Balanus improvisus* nauplii in response to a 480-nm light stimulus applied at 2.6 to 5.1 sec: (A) 27 W/m²; (B) 0.27 W/m²; (C) 0.027 W/m²; (D) 0.0027 W/m²; (E) 0.00027 W/m². The number of naupliar paths analyzed (N) is indicated. Initial and recovery represent "dark" periods of 830-nm sub-stage illumination only.

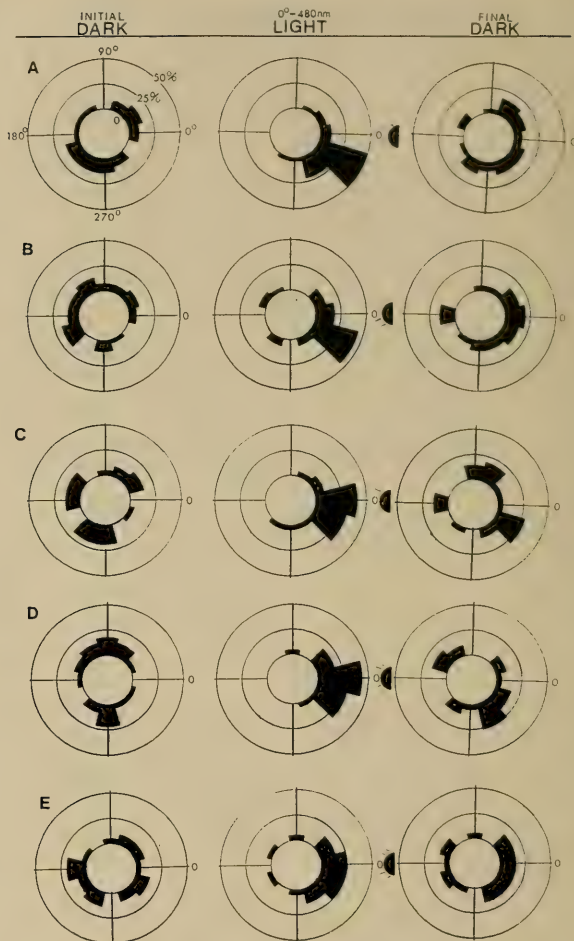


FIGURE 3. Distribution of mean direction of travel of stage II *Balanus improvisus* during initial 2.5-sec "dark" interval, 2.5 sec 480-nm light stimulus at 0°, and final 3.0-sec "dark" interval: (A) 27 W/m²; (B) 2.7 W/m²; (C) 0.27 W/m²; (D) 0.027 W/m²; (E) 0.0027 W/m². Sample numbers and "dark" condition are as in Figure 2. Significantly ($P \leq 0.05$) nonrandom distributions are indicated in Table I.

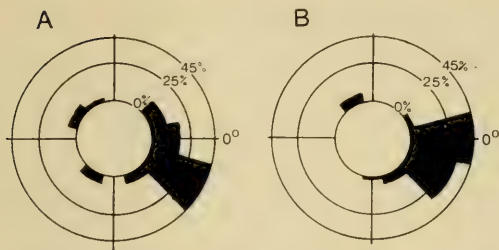


FIGURE 4. Distribution of mean direction of travel for 38 stage II *Balanus improvisus* exposed to a 480 nm, 2.7 W/m² light stimulus at 0°: (A) directional response during full 2.5-sec stimulus; (B) directional response during the latter 2.0 sec of the same stimulus.

Stage II response spectrum

Dark-adapted stage II nauplii (20° C, 15‰) were exposed to filtered light from 440 to 640 nm in 20-nm increments. Quantal intensity was balanced to approximately 0.07×10^{16} quanta m² sec at each wavelength (calculated values ranged

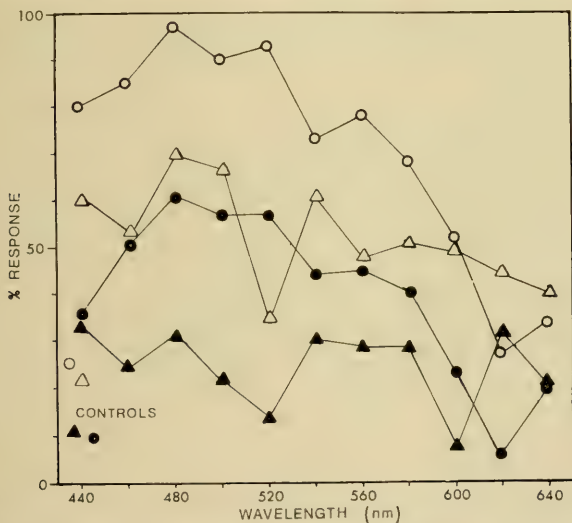


FIGURE 5. Response spectrum for positive phototaxis (% response). Open circle, stage II *Balanus improvisus*; response $\pm 45^\circ$ of light source; closed circle, $\pm 15^\circ$ of light source. Open triangle, stage VI *B. improvisus*; $\pm 45^\circ$ of light source; closed triangle, $\pm 15^\circ$ of light source. See Table II for sample numbers.

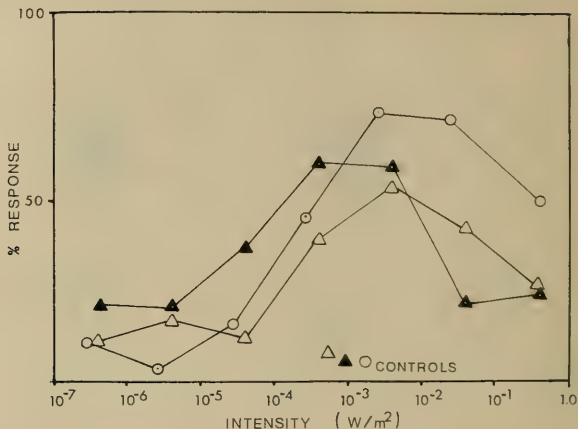


FIGURE 6. Intensity response for positive phototaxis of stage II *Balanus improvisus* at 480 nm. Closed triangle, dark-adapted at 20° C, 15‰ (N = 26-47); open triangle, light-adapted at 20° C, 15‰ (N = 53-60); open circle, light-adapted at 20° C, 30‰ (N = 22-29).

from $0.062-0.088 \times 10^{16}$). This quantal intensity was chosen because at 480-nm light it produced a clear positive phototactic response, but did not evoke a maximal response.

Analysis of DOT for nauplii indicates a broad response spectrum (Fig. 5). Increasing the $\pm 15^\circ$ "window" for positive response to $\pm 45^\circ$ produces a similar curve at response levels 30 to 40% higher. The peak percentage of positive phototaxis occurs at 480 nm ($60\% \pm 15^\circ$; $97\% \pm 45^\circ$), however similar strong responses are evident at 500 to 520 nm. A significant negative response did not occur at any wavelength.

Although peak swimming speeds were found between 460 and 520 nm, distinct differences in MLV relative to wavelength are absent. One way analysis of variance followed by the Duncan test ($P = 0.05$) yields three broadly overlapping homogeneous subsets, where the difference in the means of any two groups within a subset is not significant (Table II).

Intensity response

Light- and dark-adapted stage II nauplii (20° C, 15‰) were exposed to seven intensities of 480-nm light. In both groups, response to light was either positive or not evident; negative phototaxis did not occur significantly above random predictions (Fig. 6).

In close agreement with initial results, light-adapted nauplii exhibit a significant MLV increase at light intensities above $10^{-3} W/m^2$ (Table III). Dark-adapted nauplii significantly increase MLV above $10^{-4} W/m^2$ and, in contrast to

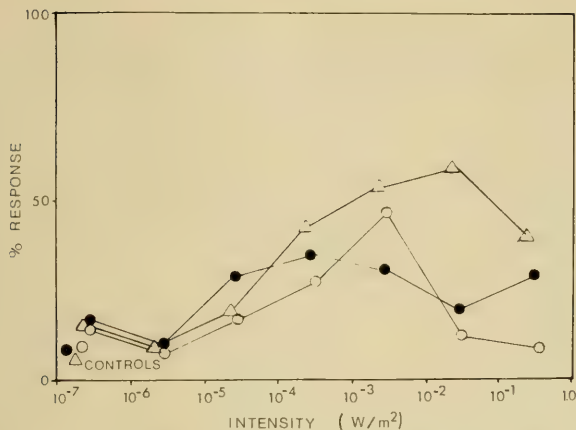


FIGURE 7. Intensity response for positive phototaxis of stage III and stage VI *Balanus improvisus* at 480 nm. Open triangle, light-adapted stage III at 20° C, 30‰ (N = 22-29); open circle, light-adapted stage VI at 20° C, 30‰ (N = 25-42); closed circle, dark-adapted stage VI at 20° C, 30‰ (N = 46-56).

light-adapted larvae, dark-adapted larvae show no increase in MLV at full intensity (Table III).

Directional response results further demonstrate the effects of dark adaptation. Light-adapted nauplii show a plateau of strong positive phototaxis from 10^{-3} to 10^{-1} W/m²; a similar pattern occurs from 10^{-4} to 10^{-2} W/m² for dark-adapted nauplii (Fig. 6).

A significant positive phototactic response is present at all intensities tested for dark-adapted larvae. For light-adapted larvae, no significant response occurs below 10^{-4} W/m². In respect to percent response and MLV, dark-adapted nauplii appear to have a somewhat "stronger" response to favorable light intensities relative to light-adapted nauplii (Fig. 6).

Some light-adapted stage II nauplii were also investigated at 30‰, 20° C, to permit comparison with later stage nauplii tested at higher salinity. A similar, but perhaps enhanced response, relative to results at 15‰ was observed (Fig. 6). The change in salinity does not alter the basic pattern of response in stage II nauplii.

Light response in later stage nauplii

Smaller numbers of stage III and stage VI nauplii were tested for response to light, in addition to stage II nauplii. The later stage *B. improvisus* larvae are likely to be carried into increased salinity water (Bousfield, 1955). Hence, these initial studies were conducted at 30‰.

Phototactic response of light-adapted stage III naupli (30‰, 20° C) is similar to stage II nauplii (Fig. 7). The control MLV of stage III nauplii tends

TABLE II

Mean linear velocities of stage II and stage IV *Balanus improvisus* nauplii in response to 2.5-sec stimuli of light at different wavelengths. Homogeneous subsets as determined by the Duncan's multiple range test ($P = 0.05$) are indicated (see Nie, et al., 1975).

Wavelength 7.0-9.0 $\times 10^{14}$ quanta/m ²	Stage II			Stage VI		
	N	MLV $\pm$ s.d. mm/sec	subset	N	MLV $\pm$ s.d. mm/sec	subset
440	26	1.75 $\pm$ 0.66	BC	35	3.72 $\pm$ 1.35	A
460	46	1.97 $\pm$ 0.77	AB	33	3.21 $\pm$ 1.19	AB
480	40	1.85 $\pm$ 0.83	ABC	35	3.36 $\pm$ 1.38	AB
500	45	2.02 $\pm$ 0.99	AB	35	3.26 $\pm$ 1.49	AB
520	45	2.13 $\pm$ 0.79	A	40	2.94 $\pm$ 1.07	AB
540	40	1.92 $\pm$ 0.62	ABC	37	2.83 $\pm$ 1.51	B
560	34	1.69 $\pm$ 0.75	BC	34	2.80 $\pm$ 1.37	B
600	50	1.56 $\pm$ 0.60	C	36	3.05 $\pm$ 1.37	AB
620	34	1.81 $\pm$ 0.60	ABC	37	3.16 $\pm$ 1.18	AB
640	55	1.78 $\pm$ 0.66	BC	48	2.89 $\pm$ 1.18	B

to be greater than stage II (Table III). Stage III nauplii show no significant increase in MLV during any light stimulus tested (Table III) therefore the photo-kinetic response seen in stage II nauplii is absent.

Both the response spectrum and responses to different intensities were tested for stage VI nauplii. Photo-responsiveness of stage VI larvae is generally less than that observed for stage II and III larvae; individual variation in nauplii is noticeably increased.

The spectral response function of stage VI nauplii using a $\pm 15^\circ$ "window" for positive photo-response yields a generally low response of about 25 to 35% at all wavelengths, except 520 and 600 nm (Fig. 5). Increasing the window to $\pm 45^\circ$ produces a curve similar to stage II with peak response at 480 nm, however, responsiveness is 20% or more below stage II results (Fig. 5). Decreased responsiveness of stage VI nauplii at 520 nm and increased responsiveness above 600 nm relative to stage II larvae is evident (Fig. 5). Unlike stage II results, increasing the window to $\pm 45^\circ$ changes the basic shape of the response curve for stage VI nauplii and is perhaps indicative of less precise orientation to the light stimulus during the 2.5-sec test interval.

The response of stage VI nauplii to different intensities of 480-nm light is most interesting in respect to dark adaptation. Light-adapted stage VI nauplii first show a significant phototactic response above 10^{-4} W/m² (Fig. 8), which is in agreement with stage II results (Fig. 7). However, unlike stage II nauplii, the positive phototaxis of stage VI nauplii rapidly diminishes above 10^{-2} W/m². Dark adaptation of stage VI nauplii, although enhancing phototaxis between 10^{-5} and 10^{-4} W/m² (Fig. 7), does not appreciably alter the threshold of response. Finally, in marked contrast to stage II (Fig. 7), dark adaptation does not decrease responsiveness of stage VI nauplii to higher light intensities (Fig. 7).

The MLV of stage VI nauplii did not vary significantly as a function of light intensity or wavelength (Table II, III). The MLV for all samples (3.05 mm/sec) was significantly above stage II and III results.

TABLE III

Mean linear velocities of *Balanus improvisus* naupliar stages II, III, and VI in response to 2.5 sec 480-nm light stimuli of different intensities. Control represents swimming speeds with 830-nm sub-stage illumination. Homogeneous subsets as determined by the Duncan's multiple range test ($P = 0.05$) are indicated for each naupliar test group (see Nie, et al., 1975).

Intensity	Dark-adapted II			Light-adapted II		
	N	MLV $\pm$ s.d.	subset	N	MLV $\pm$ s.d.	subset
6.2×10^{-1}	32	1.63 0.84	BC	60	1.91 0.81	A
$\times 10^{-2}$	33	2.22 0.96	A	59	1.92 0.88	A
$\times 10^{-3}$	26	2.11 1.10	A	58	1.77 0.65	AB
$\times 10^{-4}$	37	1.94 0.73	AB	59	1.58 0.60	BC
$\times 10^{-5}$	36	1.55 0.588	C	60	1.44 0.51	C
$\times 10^{-6}$	47	1.54 0.51	C	54	1.63 0.66	BC
$\times 10^{-7}$	39	1.41 0.57	C	53	1.54 0.53	BC
Control	40	1.52 0.55	C	55	1.48 0.49	C

Intensity	Light-adapted III			Dark-adapted VI		
	N	MLV $\pm$ s.d.	subset	N	MLV $\pm$ s.d.	subset
4.5×10^{-1}	24	2.14 0.86	A	50	2.94 1.52	A
$\times 10^{-2}$	29	2.40 0.70	A	50	3.00 1.61	A
$\times 10^{-3}$	25	2.32 0.81	A	49	3.03 1.74	A
$\times 10^{-4}$	22	2.24 0.87	A	47	3.15 1.84	A
$\times 10^{-5}$	24	2.18 0.90	A	46	2.60 1.29	A
$\times 10^{-6}$	24	2.23 0.88	A	56	3.00 1.50	A
$\times 10^{-7}$	23	2.08 0.96	A	49	3.08 1.79	A
Control	23	2.20 0.73	A	50	2.98 1.25	A

DISCUSSION

The photophysiology of zooplankton has been recently reviewed by Forward (1976b). In general, zooplankton living in coastal and fresh water tend to have their primary spectral maximum in the 500- to 600-nm region, the wavelengths best transmitted in these waters. Visscher and Luce (1928) noted maximal response of *B. amphitrite* and "*B. improvisus*" cyprids at 530 to 545 nm (although reported as *B. improvisus* cyprids, the time of collection and similarity in size to *B. amphitrite* cyprids suggest *Balanus cbeureus* as the more probable species tested (McDougall, 1943; Lang, 1979)). In more precisely controlled experiments using stage I *E. modestus* and *S. balanoides* nauplii, Barnes and Klepal (1972) found maximal spectral sensitivity between 520 and 530 nm, with a marked shoulder of strong responses at 450 to 530 nm.

Stage II *B. improvisus* nauplii have a maximal spectral sensitivity at 480 to 520 nm, somewhat shorter wavelengths than expected considering their estuarine habitat and other previous findings for barnacle larvae. Strong photo response occurs within this spectral range with maximal positive phototaxis at 480 nm (Fig. 6) and maximal MLV response to light stimuli at 520 nm (Table II).

Light response studies of stage VI nauplii are hindered by high individual variability of responsiveness; some nauplii appear highly sensitive to light stimuli while

others are unresponsive. Those stage VI nauplii which do respond show strong sensitivity to 440 to 500 nm, are curiously less sensitive to 520-nm light, and appear to be more sensitive to wavelengths at and above 540 nm relative to stage II (Fig. 6). Considerable structural and, presumably, physiological changes occur in stage VI nauplii in preparation for metamorphosis to cyprid (Walley, 1969). This includes development of compound eyes under the dorsal cephalic shield. Stage VI nauplii may have, depending on their age, only a single median naupliar eye, or two well pigmented compound eyes (Kaufmann, 1965; Lang, 1979). Since the larval samples tested in this study included all phases of stage VI development, variability of responsiveness is not unexpected. Increased sensitivity to higher wavelengths is perhaps related to development of compound eyes; studies on specific phases of stage VI development and, more importantly, the cyprid stage, are needed to verify this hypothesis.

Contrasting qualitative observation of photokinetic responses in barnacle larvae have been reported in the literature. Ewald (1912) noted a characteristic sinking reaction in stage II specimens of *Balanus perforatus* following a sudden dark-to-light transition. Similarly Crisp and Ritz (1973) saw decreased activity in *S. balanoides* cyprids exposed to sudden light increase and conversely, increased activity following light intensity reduction. Essentially opposite reactions were observed in our laboratory for stage II *Balanus venustus*, *B. improvisus*, and *B. amphitrite*; MLV sharply increased following sudden exposure to bright white light and MLV sharply decreased following removal of the light stimulus (Lang, *et al.*, 1979). Photokinetic responses were found absent in stage II nauplii of *S. balanoides* and *E. modestus* (Crisp & Ritz, 1973) and in *Chthamalus fragilis* (Lang, *et al.*, 1979).

The present study shows that stage II nauplii of *B. improvisus* consistently increases MLV following exposure to a specific range of light intensities at 480 nm, the range being determined, in part, by the initial state of naupliar light adaptation. Dark-adapted nauplii show photokinetic response at reduced light intensities relative to light-adapted nauplii. The upper intensity, if any, which inhibits increased MLV in light-adapted nauplii was above the maximum tested (27 W/m^2). An increase in MLV appears to represent a second aspect in stage II light response. At detectable, but suboptimum intensities, stage II nauplii exhibit positive phototaxis but show no significant change in MLV. Only within a narrower range of light intensities inducing maximal response does MLV increase. A similar change in MLV does not occur in light-adapted stage III nauplii or light- and dark-adapted stage VI nauplii. Results indicate at least two possibilities; either stage II nauplii exhibit a "stronger" response to light incorporating both directional and kinetic reactions or, later stages normally swim at or near their potential MLV and are incapable of further MLV increase in response to light stimuli.

Earlier studies (Lang, *et al.*, 1979) showed that *B. improvisus* nauplii will briefly stop or reduce MLV when a strong white light source was removed. Removal of the 480-nm light stimuli in present studies did not induce this response. We assume this response occurs only with more intense and/or longer light stimulation.

The level of previous adaptation to light has been shown to significantly effect the photoresponse of zooplanktons (Forward, 1976b). Crisp and Ritz (1973)

demonstrated relative reductions in barnacle naupliar light responsiveness following exposure to strong light. Continual exposure to light of sufficient intensity will induce a photonegative response in barnacle nauplii (Groom and Loeb, 1890).

A change in responsiveness to light following dark-adaptation is clearly seen here in *B. improvisus* stage II nauplii. The threshold for positive phototaxis in light-adapted nauplii is at least an order of magnitude greater relative to dark-adapted naupliar responses. Conversely, light-adapted phototaxis is significantly less suppressed at higher light intensities (Fig. 6). Positive phototaxis for at least 10% (above control) of dark-adapted stage II nauplii tested was seen at 6.2×10^{-7} W/m² at 480 nm. A consistent, strong response (*i.e.*, 30% or more of the test population) occurred above 10^{-5} W/m². These values bracket the same order of magnitude for light responsiveness reported by Barnes and Klepal (1972) and Crisp and Ritz (1973) for three barnacle species of naupliar stages I–II.

A similar clear shift in responsiveness of light- and dark-adapted stage VI nauplii was not seen. In particular, dark-adapted nauplii remained equally or more responsive to higher light intensities as compared to light-adapted nauplii (Fig. 7). Cyprid larvae of some barnacles are known to exhibit at least two fundamental light responses: orientation at fixation and shade-seeking during exploration (Crisp and Ritz, 1973). As with spectral results, we would eventually like to demonstrate whether idiosyncrasies in stage VI light behavior relate to light responses in *B. improvisus* cyprids.

The degree of light-adaptation, although it affects the intensity sensitivity of *B. improvisus* nauplii, does not alter the basic types of response. Under the stable and presumably favorable salinity-temperature conditions tested, the immediate reaction of *B. improvisus* to light stimulation is either positive phototaxis or no response. A characteristic "shadow response" with negative phototaxis seen in light-adapted brachyuran zoea (Forward, 1976a, 1977) tested under similar light stimuli is absent.

In his classic field study, Bousfield (1955) showed that stage II *B. improvisus* nauplii maintained an average water column position near the surface, while later naupliar stages were found at progressively lower average depths. The strong positive photo response of stage II nauplii and marked decline of photo responsiveness in stage VI nauplii seen here correlates well with these field observations. However, a discussion of ecological implications of larval photobehavior is best deferred until further studies are complete.

This study most importantly demonstrates a new research technique. Video-computer systems decidedly enhance the ability to convert visual (video) records into quantified data. With this ability comes a new potential to initiate studies on locomotory behavior involving various parameters and to measure responses with a resolution previously not readily obtainable.

Additional software development for analysis of phototactic responses was provided by Dr. Robert Wilson. Technical assistance for Bugwatcher operations was provided by Dr. John Greaves. Martha Marcy and Sally Lawrence contributed significant assistance in larval maintenance and data analyses.

SUMMARY

A video-computer behavioral analysis system—the "Bugwatcher"—was found to be capable of rapidly and accurately analyzing the phototactic movements of stage II, III, and VI nauplii of the barnacle *Balanus improvisus*. Under the test conditions these larvae only display a positive phototactic response; a negative response was not observed. The response spectrum of dark-adapted stage II shows a plateau of strongest positive phototaxis between 480 and 520 nm with about 60% of test larvae swimming $\pm 15^\circ$ toward the light stimulus. In contrast, stage VI nauplii are generally less phototactic. The response spectrum changes to have a depression at 520 nm and enhanced responsiveness to longer wavelengths of 540 to 580 nm.

Responsiveness to different light intensities changed upon light adaptation. Upon stimulation with 480 nm lights, dark-adapted stage II nauplii show a significant positive phototaxis at 6.2×10^{-7} W/m². Peak response occurs between 10^{-4} and 10^{-2} W/m². A significant increase in mean linear velocity (MLV) accompanies the maximal phototactic response. In contrast, upon light adaptation, stage II nauplii show a significant positive phototaxis only to intensities above 10^{-4} W/m², 480 nm. Peak response occurs between 10^{-3} and 10^{-1} W/m². A significant increase in MLV occurs from about 10^{-3} to at least 27 W/m². Light-adapted stage III nauplii show intensity sensitivity similar to stage II nauplii; however, no increase in MLV occurs during light stimulation. Stage VI nauplii show a reduced percent phototactic response at all intensities as compared to stage II–III. Light- or dark-adaptation does not result in clear shifts in intensity sensitivity as evident in stage II responses. Differences in stage VI naupliar light responses may be related to impending metamorphosis to cyprid. A study of cyprid light response is needed to clarify this.

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THE EFFECTS OF EYESTALK, LEG, AND UROPOD REMOVAL ON THE MOLTING AND GROWTH OF YOUNG CRAYFISH, *PROCAMBARUS CLARKII*

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The effects of eyestalk removal on molt have been studied in decapod crustaceans (Brown and Cunningham, 1939; Abramowitz and Abramowitz, 1940; Smith, 1940), but few crustaceans have been studied during several consecutive molts, probably because of a high mortality. According to Abramowitz and Abramowitz (1940), however, the removal of the eyestalk itself is unlikely to be the reason for the mortality, because some of their blinded crabs survived for 11 weeks after the removal of eyestalks.

In our preliminary experiments (unpublished), 14 of 90 eyestalkless young crayfish survived after more than 10 molts, though the rest died within 4 months. In most cases, the eyestalkless crayfish died at the time of molt or within 1 or 2 days after the molt, probably because of bacterial infection at the time of molt. If this is so, a high mortality might be avoidable to some extent by keeping the container water clean, without disturbing the molting animals.

The molt cycle in Crustacea is thought to be regulated by two hormones, a molt hormone and a molt-inhibiting hormone. It is believed that the X-organ in the eyestalk produces the molt-inhibiting hormone, which is then stored in the sinus gland. Eyestalk removal, therefore, shortens the following intermolt cycle (Brown and Cunningham, 1939; Smith, 1940). The molt-inhibiting hormone inhibits the activity of the Y-organ, which secretes the molt hormone (Passano, 1960). When the eyestalk is removed the Y-organ is no longer inhibited. If the larvae of the prawn *Palaemonetes kadiakensis* are destalked at metamorphosis, they grow larger in size than the untreated ones, without causing any alteration on the duration of the larval instars (Hubschman, 1963).

It is known that leg removal causes precocious molt in land crabs, *Gecarcinus lateralis* (Skinner and Graham, 1970), freshwater shrimp, *Palaemonetes kadiakensis* (Stoffel and Hubschman, 1974), and crayfish, *Procambarus clarkii* (Bittner and Kopanda, 1973). Skinner and Graham (1972) hypothesized that severing a critical number of leg nerves stimulates the precocious molt.

In the present study, the molt interval and the growth rate of the crayfish were studied during five consecutive molts when the eyestalks, the legs or the uropods were removed.

MATERIALS AND METHODS

Specimens of the crayfish, *Procambarus clarkii*, used in this work were collected from ponds in the suburbs of Yamagata. Only crayfish of 8 to 12 mm in length (the length was measured from the tip of rostrum to the hind margin of cephalothorax carapace) that molted once in the laboratory (initial molt) were

chosen as experimental material. They were kept separately, first in polypropylene containers (Lustro ware of Boden Co., $70 \times 85 \times 45$ mm), then in large containers ($105 \times 120 \times 53$ mm) containing dechlorinated tap water at 22 to 23° C when they grew to more than 15 mm in carapace length. They were kept under a photoperiodic light condition of 14-hr light and 10-hr dark. The animals were fed fish food pellets and fallen dead leaves of persimmon. The dead leaves were effective in keeping the animals healthy. When water in the containers was renewed each day, the crayfish were immersed in 1.3% NaCl solution for 20 to 30 sec to prevent infection by bacteria or protozoa.

The crayfish were classified into four experimental groups: (1) untreated intact crayfish as control; (2) crayfish from which a pair of eyestalks was removed; (3) crayfish from which three pairs of the second, third and fourth walking legs were removed, and (4) crayfish from which a pair of uropods was removed. Each group consisted of 23 individuals that had just finished the initial molt in the laboratory, and every operation was performed on the day following this initial molt. The organs were cut off at their bases with scissors. After the third molt following the operation, the regenerated legs or uropods were removed again. Carapace length was measured two days after each molt.

RESULTS

Molting

Molting rate was recorded daily for 230 days after the initial molt. The results are illustrated in Figure 1A-D. The crayfish which died during the course of the experiments were excluded from the data. Four crayfish from the control group, one from the legless group, and one from the uropodless group failed to complete the fifth molt even after 230 days. Since these animals had a reduced fecal output, their failure to molt may have been the result of factors other than hormonal or nervous controls.

Untreated crayfish (controls). Fourteen individuals completed the fifth molt, but three molted four times and one completed only the third molt. The average time required for 50% of the individuals to reach each consecutive molt (T_{50}) was 17 days for the first molt, 50 days for the second, 87 days for the third, 128 days for the fourth, and 164 days for the fifth, after the initial laboratory molt.

TABLE I
The days of the intermolt cycles (molt to molt) in crayfish, Procambarus clarkii.

Molt	Control (14)	Eyestalkless (11)	Legless (20)	Uropodless (20)
Initial to 1st	19.6 ± 2.7	6.5 ± 0.2	14.1 ± 1.0	19.7 ± 2.3
1st to 2nd	23.8 ± 2.9	7.1 ± 0.3	12.6 ± 0.9	26.9 ± 2.9
2nd to 3rd	33.6 ± 4.2	7.5 ± 0.2	31.5 ± 3.8	33.6 ± 4.2
3rd to 4th	31.3 ± 3.1	8.8 ± 0.2	21.1 ± 1.6	29.9 ± 2.6
4th to 5th	43.2 ± 6.0	9.2 ± 0.4	35.2 ± 4.6	39.0 ± 3.4

The number of individuals is in parentheses. Data were obtained from crayfish which had completed five consecutive molts. The standard error of the mean is shown.

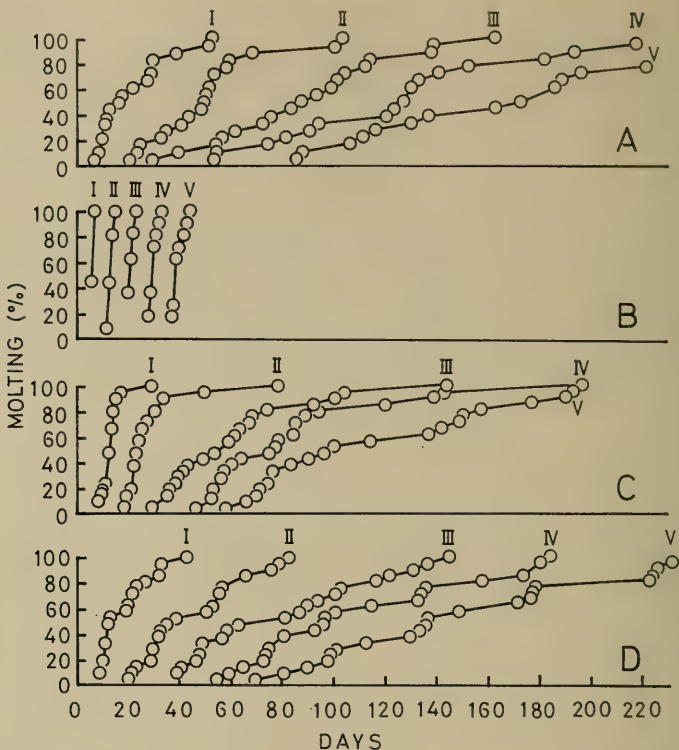


FIGURE 1. Molting percentage of surgically treated crayfish, specimens of *Procambarus clarkii*, at five consecutive molts after the initial laboratory molt. A, untreated crayfish (control); B, both eyestalks removed; C, three pairs of walking legs removed; D, both uropods removed. In the groups where legs or uropods were removed, the regenerated legs or uropods were removed again one day after the third molt. Roman numerals I, II, III, IV and V, show the 1st, 2nd, 3rd, 4th, and 5th molt, respectively.

Large variation, however, was observed among the individuals. For example, in the first molt the earliest crayfish molted on the seventh day but the last crayfish molted on the fifty-third day. As clearly shown in Figure 1, this variation became more pronounced in the later molts.

Eyestalkless crayfish. Twelve of 23 treated crayfish died between the third and fifth molts. The remaining 11 completed all five consecutive molts within 43 days. T_{50} from the first to the fifth molt was 6 (35.3% compared to the value of

control animals), 13 (26.0%), 20.5 (23.6%), 29 (22.7%), and 39 (23.8%) days, respectively. The first molt occurred on the fifth day and all the treated individuals molted by the eighth day. All 23 crayfish completed the second molt between 6 and 8 days after the first molt and finished the third molt between 7 and 8 days after the second molt. Two animals, however, died on the first and fifth day respectively, after the third molt.

The surviving crayfish completed the fourth molt by the intermolt cycle of 7 to 12 days. Seven animals died within two days after the fourth molt. The 14 survivors completed the fifth molt by the intermolt cycle of 8 to 12 days. Three died at the time of the fifth molt.

Legless crayfish. Twenty of 21 animals completed five consecutive molts (one animal completed only four). T_{50} from the first to the fifth molt was 13 (76.5% of the control value), 24 (48.0%), 55 (63.2%), 77 (60.2%), and 99 (60.4%) days, respectively.

A few animals had longer molt cycles than the others. Roughly speaking, the operated animals could complete five molts during the time in which the untreated crayfish completed four molts. The walking legs regenerated to normal size after the second molt. Two crayfish died; one at the time of its third molt and another at its fourth molt.

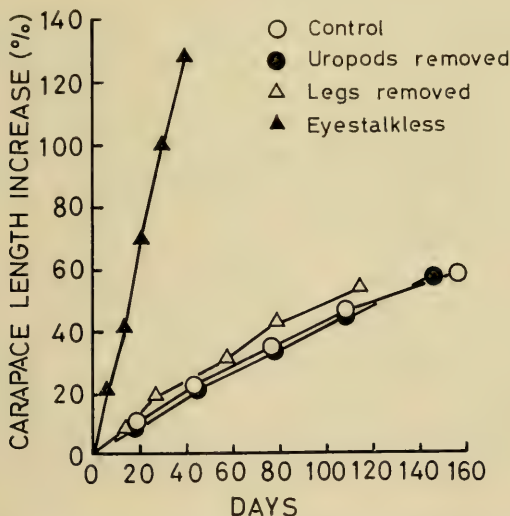


FIGURE 2. The average percentage of increase in carapace length of specimens of the crayfish, *Procambarus clarkii*, to the original length after each of the five consecutive molts. Each point represents the mean days required for each molt following the initial laboratory molt.

TABLE II

The average carapace length (mm) after each molt in crayfish, Procambarus clarkii.

	Initial	1st	2nd	3rd	4th	5th	Mean
Control	11.1 ± 0.2	12.3 ± 0.3 (10.5)	13.5 ± 0.4 (10.4)	14.9 ± 0.5 (10.2)	16.1 ± 0.5 (8.2)	17.5 ± 0.6 (8.5)	(9.6)
Eyestalkless	11.5 ± 0.3	13.9 ± 0.3 (20.7)	16.3 ± 0.4 (17.5)	19.5 ± 0.5 (19.8)	22.8 ± 0.6 (16.8)	26.2 ± 0.7 (14.7)	(17.9)
Legless	11.2 ± 0.3	12.1 ± 0.3 (8.1)	13.3 ± 0.3 (10.0)	14.6 ± 0.4 (10.2)	15.9 ± 0.4 (8.6)	17.1 ± 0.5 (7.8)	(8.9)
Uropodless	11.6 ± 0.3	12.7 ± 0.3 (8.9)	14.1 ± 0.5 (11.3)	15.5 ± 0.6 (9.7)	16.8 ± 0.7 (8.6)	18.3 ± 0.9 (8.6)	(9.4)

The numerals in parentheses are the average percentage of increased carapace length for each molt.

Uropodless crayfish. Twenty of 21 animals completed five consecutive molts, one animal staying at the fourth molt during the experimental period. T_{50} from the first to the fifth molt was 12 (70.0% of the control value), 39 (78.0%), 73 (83.9%), 96 (75.0%), and 136 (82.9%) days, respectively. Regeneration of the uropods was observed on all crayfish after the second molt. Two crayfish died: one at the first molt and another at the third molt.

Growth rates

The mean values of growth rate for crayfish which completed five molts were measured after each of five consecutive molts with special reference to the mean value of the initial carapace length, which was measured after the initial laboratory molt (Fig. 2). As is clear from Figure 2, the rate of increase of carapace length of the eyestalkless group is more than six times greater than that of the control group.

The percentage increases in carapace length from between each premolt and corresponding postmolt stage are shown in Table II. The average percentage of increase in carapace length per molt was 9.6% for untreated group, 17.9% for eyestalkless group, 8.9% for legless group, and 9.4% for uropodless group.

DISCUSSION

The results suggest that the removal of eyestalks, legs, or uropods stimulates molt in the crayfish, *Procambarus clarkii*. The results support the findings of other authors studying eyestalkless crayfish, *Cambarus clarkii* (Smith, 1940), fiddler crabs, *Uca pugilator* (Abramowitz and Abramowitz, 1940) and land crabs, *Gecarcinus lateralis* (Skinner and Graham, 1972); and walking legless crayfish, *Procambarus clarkii* (Bittner and Kopanda, 1973), freshwater shrimp, *Palaemonetes kadiakensis* (Stoffel and Hubschman, 1974) and land crabs, *Gecarcinus lateralis* (Skinner and Graham, 1970). Brown and Cunningham (1939) and Smith (1940) discussed the possible mechanisms concerning the effects of eyestalk removal on the molt, suggesting that the eyestalks contain the inhibiting hormone which delays molting.

Abramowitz and Abramowitz (1940) found that in fiddler crabs both molt and growth were stimulated by eyestalk removal. In our experiments, we found that the average growth rate of carapace length in the eyestalkless crayfish after every molt was about twice that of the intact animals, and that the duration of the molt

cycles of the former was about one fourth of that of the latter. From these data, the mean weight increase of eyestalkless animals was about 15 times larger than the corresponding weight of the control animals 40 days after the operation. On the other hand, the molts of both the leg- or uropod-removed crayfish were stimulated, but the carapace length growth rate after every molt was less than that of the controls. Thus, it is clear that growth was accelerated by eyestalk removal, and that the secretion of molting hormone was induced by the growth of the body.

Weis (1976) found that the removal of seven legs from a fiddler crab did not result in a significant increase in carapace width after molt and regeneration. Krishnakumaran and Schneiderman (1970) found that ecdysterone did not increase DNA synthesis in the epidermis, muscles, nerve cells, and connective tissue of crayfish, *Procambarus clarkii*, although it induced molting. In the present experiments, the carapace length increased after molt in crayfish whose legs or uropods were removed, but the rate of increase was less than that of the untreated control.

It may be safely concluded that the removal of legs or uropods has the same effects on molt and growth in crayfish. This would support the hypothesis of Skinner and Graham (1972) that molt inhibitory factors do not exist in the limbs of Crustacea and that precocious molt is stimulated by the severing of a critical number of nerves. Stoffel and Hubschman (1974) pointed out that the loss of several walking legs stimulates the neurosecretory cells of the X-organ via nervous impulses to stop releasing the molt-inhibiting hormone. According to Holland and Skinner (1976), the removal of one or more limb buds of *Gecarcinus lateralis* inhibited growth of the remaining limb buds until re-regenerates reached an appropriate size. In the present experiment, both legless or uropodless crayfish molted before they grew in body size sufficient for molt. Therefore, if the crayfish is missing legs or uropods, it may be possible that the regenerating buds grow faster than all the other parts of the body, and the regenerating buds stimulate the release of molt hormone. The intensity of stimulus may depend, to some extent, on the number of buds, or on the surface of the cut ends or organs. It is known that land crabs missing many legs prepare for molt sooner than those which are missing one or two legs (Skinner and Graham, 1970).

It has been reported that the mortality is high in destalked crustaceans (Abramowitz and Abramowitz, 1940; Smith, 1940; Skinner and Graham, 1972). According to Abramowitz and Abramowitz (1940), the viability is concerned in some way with the eyestalks. In general, death during molt by intact Crustacea is common. In the present experiments, five crayfish of the control group, two of the legless group, and two of the uropodless group died at the time of molt or within a day after their molt. In the eyestalkless group, twelve individuals died at the time of molt or within a few days after their molt. The mortality of the eyestalkless group, therefore, is higher than the other groups. It may be possible that the eyestalkless crayfish grow too rapidly to prepare properly for molt and this leads to failure at molt.

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SUMMARY

1. Removal of a pair of eyestalks induces precocious molt and accelerates the growth of the crayfish, *Procambarus clarkii*.
2. Removal of three pairs of walking legs or a pair of uropods induces precocious molt without any effects on the growth of the body.
3. The average time required for 50% of the individuals to reach the fifth molt after the initial molt is 39 days for eyestalkless crayfish, 99 days for legless, and 136 days for uropodless crayfish. The untreated crayfish (controls) required 164 days to attain the fifth molt.
4. The average percentage of increase in carapace length at the time of the fifth molt is 9.6% for the untreated crayfish, 17.6% for eyestalkless, 8.9% for legless, and 9.4% for the uropodless group.
5. The mortality during the approximately eight-month experimental period was 5:23 for the untreated group, 12:23 for the eyestalkless group, 2:23 for the legless group and 2:23 for the uropodless group. The eyestalkless crayfish were healthy until the third molt, but experienced great mortality at the time of the fourth and fifth molt. The failure of molt in eyestalkless crayfish may be due to too rapid increase in the body size, impairing preparations for molt.

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EVOKED RESPONSES TO ELECTRICAL STIMULATION IN THE COLONIAL HYDROID *CLAVA SQUAMATA*: A CONTRACTION PULSE SYSTEM¹

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Electrical activity has been recorded from several hydrozoans (phylum Cnidaria) representing two distinct classes of polyps—those which produce recurring behavioral events of spontaneous origin, for example, *Corymorpha* (Ball, 1973; Ball and Case, 1973); *Hydra* (Josephson, 1967; Josephson and Macklin, 1969; Passano and McCullough, 1962, 1963, 1964, 1965; Rushforth, 1966, 1971; Rushforth and Burke, 1971); *Millepora* (deKruif, 1976); *Obelia* (Morin and Cooke, 1971a, b, c); *Tubularia* (deKruif, 1977; Josephson, 1962, 1965, 1974; Josephson and Mackie, 1965; Josephson and Rushforth, 1973; Josephson and Uhrich, 1969); and those which are behaviorally quiescent, e.g. *Cordylophora* (Josephson, 1961b; Mackie, 1968); *Hydractinia* (Stokes, 1974a, b) and *Proboscidiactyla* (Spencer, 1974). Despite these obvious behavioral differences, a review of these studies (Rushforth and Stokes, 1978) showed that there is at least one element of electrically evoked activity which is common to all species of both classes. These common elements are the large (0.5–15 mV), slow conducting (2–20 cm sec), long duration (20–500 msec) potentials which are correlated with contraction of whole or isolated parts of a polyp. Examples include the Josephson Pulses (JPs) of *Cordylophora*, Hydranth Pulses (HPs) and Neck Pulses (NPs) of *Tubularia*, Stalk Pulses (SPs) and Hydranth Pulses (HPs) of *Corymorpha*, Contraction Pulses (CPs) or Contraction Bursts (CBs) of *Hydra*, Symmetrical Contraction Pulses (SCPs) of *Hydractinia*, Contraction Pulses (CPs) of *Millepora*, Contraction Pulses (KPs) of *Obelia* and the Tentacle Contraction Pulses (TCPs) and Colonial Pulses (CPs) of *Proboscidiactyla*.

It has been proposed (Rushforth and Stokes, 1978) that these pulses are homologous representations of a fundamental conducting system termed the 'Contraction Pulse System' (CPS). This system functions to activate muscles of widespread distribution, or isolated blocks of muscles. In an effort to strengthen this proposal, we have investigated the evoked responses of the colonial gymnoblastic hydroid, *Clava squamata*. The purpose of the present study was to examine the electrical activity of this behaviorally quiescent species.

MATERIALS AND METHODS

Collection and maintenance of animals

The Supply Department of the MBL, Woods Hole, provided all animals for this study. Only colonies growing on *Ascomophyllum* were used. Colonies in which the

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polyps appeared unhealthy or damaged, or in which the alga had begun to decay, were discarded. All specimens were placed in an aerated, refrigerated (14° C) Instant Ocean System containing sea water, and were fed daily on newly hatched *Artemia* nauplii. Collected colonies were studied within 2 to 3 days. Glass microscope slide cultures of *Clava* were also made by dissecting two to three interconnected polyps from a colony and attaching them to the slide by fine surgical thread. Such cultures were maintained under conditions similar to those of the freshly collected colonies. New stolons grow out from the transplant, adhere to the glass slide and give rise to new polyps. After about 1 week the original transplant and the surgical thread can be removed, leaving only new growth attached to the slide.

Most experiments used whole colonies on their algal substrate. The alga was trimmed to about 2 to 3 cm length and pinned to the Sylgard bottom of a dish containing 500 ml of sea water. The temperature of the sea water in the dish was also maintained at 14° C by an outer jacket of circulating, refrigerated water. The attached hydroids did not appear to be damaged by this process and survived for several days under such conditions, when appropriately fed. In certain experiments single polyps were used. These polyps were excised from the colony by means of iridectomy scissors at the junction between polyp and stolon.

Stimulation and recording techniques

Suction electrodes were used for recording and stimulation. Plastic Tygon or polyethylene tubing was flame heated and drawn to diameters appropriate for attachment to a specific region of the polyp or stolon, usually 50 to 100 μ m. The tubing was then squarely and evenly cut to insure minimal damage to the soft tissue. Glass electrodes modeled after those described by Josephson (1967) were used in experiments on isolated polyps. These electrodes have a bell-shaped tip in which the polyp can be held without damaging the tissues. Both kinds of electrodes can be attached by means of mild suction obtained by manipulation of a hypodermic syringe at the opposite end of the tubing or glass. Micromanipulators served to facilitate movement and placement of the electrodes. Both stimulation or recording could be achieved through these suction electrodes, and in either case, the indifferent electrode was a coil of chlorided silver wire placed in the experimental dish. Electrical stimuli were single or repetitive pulses delivered through a stimulus isolation unit. Conventional capacitor coupled amplifiers and display devices were used.

RESULTS

General organization of the colony

Clava may be found at Woods Hole, Massachusetts in association with an intertidal brown alga, *Ascophyllum*. It is generally located at the branch points of the alga or within damaged flotation sacs, and only rarely along the lengthily exposed parts of the stem. Occasionally one can find *Clava* on rocks or wharf pilings. Colonies growing on *Ascophyllum* are comprised of upwards of 50 to 100 monomorphic polyps; the hydranth of each bears a naked hypostome and terminal

mouth, and some 20 to 30 filiform tentacles. The tentacles are uniformly distributed over the hypostomal region and do not form a distinct ring or rings. The lengthy stalk region below the hypostome is naked in immature polyps. Gonophores bud off from this stalk region 1 to 2 mm below the most proximal tentacles in mature polyps. The polyps vary in length, from about 0.5 to 1.5 cm, and are joined basally by a coenosarc of interconnecting stolons. The sexes are separate and a colony is comprised of all male or all female polyps, probably originating from a single planula. Occasionally, as reported by Föyn (1927), male and female colonies will occur together in the same clump.

General behavior and responses to mechanical stimuli

Spontaneous and rhythmically recurring behavior of individual polyps or groups of polyps does not occur under uniform conditions of illumination in *Clava*. Polyps may bend slowly or reorient individual tentacles, but the general observation is prevailing quiescence. Endogenous pacemaker systems like those responsible for the rhythmic behavior of *Tubularia* (Josephson, 1962; Josephson and Mackie, 1965) or *Hydra* (Passano and McCullough, 1962, 1965) do not appear to be present in *Clava*.

Mechanical stimulation of a polyp may induce weak or vigorous polyp activity depending on the intensity of the stimulus. Pinching an individual tentacle can result in contraction of that tentacle alone; contraction of the tentacle and movement of the hydranth towards the stimulated side; or contraction of the entire polyp. Contraction of the polyp may be graded, but in most cases, particularly colonies growing on *Ascophyllum*, it was not observed to spread to neighboring polyps. In rare cases, vigorous pinching of the hydranth resulted in a vigorous contraction of the polyp, and that contraction spread in a graded fashion to the neighboring polyps. Pinching a stolon also can elicit the same response; however, in no case were more than three polyps involved in this coordinated response.

Long-term electrical recordings from individual polyps show that electrical activity does not occur in *Clava* in the absence of behavioral events. Furthermore, many complex behaviors, particularly those associated with feeding, occur in the complete absence of detectable electrical events. Other complex responses induced by certain amino acids are associated with the generation of electrical potentials; however their role in feeding, if any, is not known.

Following mechanical stimulation of a *Clava* polyp, either single or multiple pulses can be recorded with a short latency (less than 400 msec) which correlate with the visual observation of overall polyp contraction. Weak pinching of any part of a polyp produces single electrical pulses with each contraction, whereas vigorous stimulation produces multiple electrical pulses and a more vigorous polyp contraction. These same results can be obtained by mechanical stimulation of the interconnecting stolons.

Electrical stimulation-correlated behavior and electrical activity

The electrical responses in this study have been recorded extracellularly from rather large blocks of tissue comprised of ectoderm, mesoglea and endoderm.

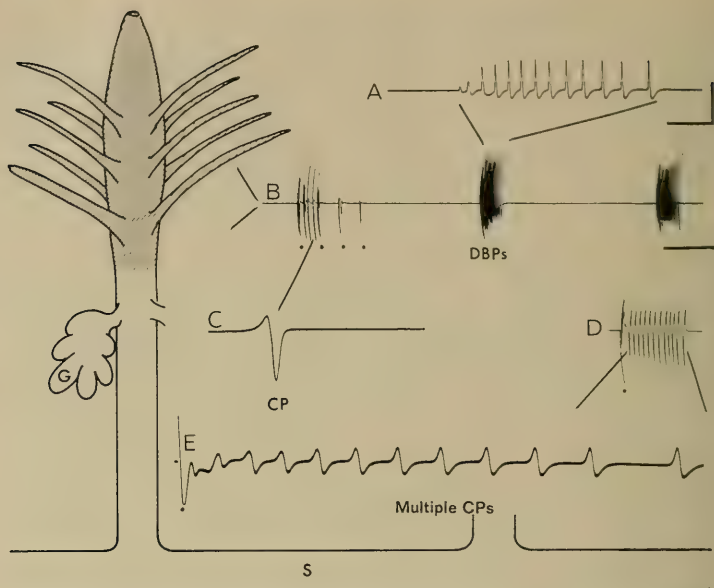


FIGURE 1. *Clava* polyp schematic showing representative kinds of evoked electrical pulses. A, delayed burst potentials similar to those shown in record B, but recorded at faster time base. Stimulus not indicated. B, a sequence of CPs and two bursts of DBPs evoked by four stimuli (dots beneath recording) applied to the burst generator region (hatched region). C, a typical CP evoked by stimulation of any part of the body column or tentacles. D, typical record of multiple CPs recorded from the polyp stalk. E, multiple CPs similar to those recorded in D, but at a faster time base. Dot indicates stimulus artifact. G, gonophores, S, stolon; Vertical scale A applies also to B, C, D, E, $=0.5$ mV; Horizontal Scale A applies also to C, E, $=0.6, 0.2, 0.3$ secs, respectively; Horizontal Scale B applies also to D $=5.0$ sec.

Positive identification of the underlying morphological source of electrical signals generated by these tissues is difficult and hence, for the moment, we cannot identify these events as neural, muscular, or epithelial. Instead the neutral term "conducting system" (Josephson and Mackie, 1965) will be used to define the substrates of these electrical events.

Two distinct kinds of electrical activity can be recorded from *Clava* depending on the stimulus site. Evidence presented here and in a subsequent report (Rushforth and Stokes, in preparation) indicates that these two events represent activity in separate conducting systems. One of these conducting systems is activated only by direct stimulation of the hydranth distal to the gonophore stalks (Fig. 1, hatched region). The electrical events (Figs. 1A, B) occur as a burst or a

TABLE I

Characteristics of electrically stimulated contraction pulses in Clava: mean $\pm$ s.e.

Pulse type	Latency (msec)	Interpulse interval (msec)	Pulse duration			Pulse amplitude mV	Conduction velocity cm/sec
			Rise time (msec)	+ve to -ve Peak (msec)	Total (msec)		
A. Single contraction pulses	331 $\pm$ 109 (3)*		74 $\pm$ 26 (2)	48 $\pm$ 11 (4)	144 $\pm$ 20 (4)	0.53 $\pm$ 0.03 (4)	2.6 $\pm$ 0.2 (11)
B. Multiple contraction pulse bursts	330 $\pm$ 70 (2)	371 $\pm$ 19 (9)	73 $\pm$ 6 (2)	50 $\pm$ 7 (9)	140 $\pm$ 8 (9)	0.61 $\pm$ 0.10 (9)	2.1 $\pm$ 0.3 (5)

* Values in parentheses = N.

program of multiple bursts of pulses after a long latency ($\bar{x} = 19.2 \pm 1.4$ sec, $N = 18$) and are referred to as Delayed Burst Potentials (DBPs). A burst of DBPs on an expanded time scale is shown in Figure 1A. In no case have DBPs been initiated by stimulation of stolons or the stalk region proximal to the gonophores. DBPs correlate with a synchronized depression of the tentacles and, at least to the first few bursts of a program, with a symmetrical shortening of the body stalk. The stalk contraction element is not apparent after the first few bursts of a program. Also, the pulses within a burst appear much more homogeneous following loss of an observable stalk contraction. The conducting system underlying these bursts of pulses, called the Delayed Burst System (DBS) is the subject of a subsequent report (Rushforth and Stokes, in preparation).

A second type of electrical event can be evoked by stimulation of the polyp at any location—tentacles, hypostome, body column or base. Characteristically, these potentials are single (Figs. 1B, C) or multiple pulses (Figs. 1D, E), which are similar in all respects to those evoked by mechanical stimulation. These potentials

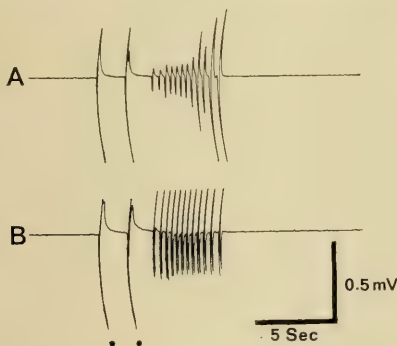


FIGURE 2. Facilitation of CP potentials within a "burst", recorded at two sites (A and B) on a single polyp stalk. Two stimuli were applied (dots) adjacent to site B.

are correlated with a symmetrical, often localized contraction of the polyp and are thus referred to as Contraction Pulses (CPs). The conducting system underlying the CPs is referred to as the Contraction Pulse System (CPS). The threshold for activation of the CPS is generally below that for the DBS. Both single and multiple CPs appear to originate within the tissues beneath the stimulating electrode and have a short latency (about 300 msec) in comparison to the DBPs. Characteristics of the electrical pulses associated with single and multiple CPs are presented in Table I. In summary, both single CP events and individual CPs of a multiple sequence are nearly identical with respect to pulse rise time (75 msec), positive to negative peak (50 msec), total pulse duration (140 msec) and pulse amplitude (0.5–0.6 mV). The refractory period for single CPs was determined to be about 200 to 250 msec, and the interpulse intervals for multiple CPs are on the average about 370 msec. As shown in Figure 1E, the interpulse intervals tend to elongate towards the end of a CP burst. CPs are generally biphasic, although triphasic CPs have also been recorded, usually following the first stimulus of a regime of stimuli given to a fully expanded polyp.

Both single and multiple CPs are nonpolarized conducted events (Table I). The conduction velocity was determined by recording from two Tygon suction electrodes attached to the same side of the polyp while stimulating through a glass holding electrode. From these records, the time delay between negative peaks of the electrical event recorded at the two sites and the measured distance between the recording electrodes were used to compute the conduction velocity. The mean conduction velocity for single CPs is 2.6 cm/sec ($N = 11$) and for multiple CPs 2.1 cm/sec ($N = 3$). For three animals, conduction velocities for single CPs were determined for both the proximal and distal directions. No significant differences were observed ($\bar{x} = 2.3 \pm 0.1$ distally and $\bar{x} = 2.2 \pm 0.3$ proximally). The mean conduction velocity for DBPs is 9.8 cm/sec ($N = 7$).

The responses of a single polyp to electrical stimulation appear to be graded with stimulus intensity. A single threshold shock to a tentacle may result only in contraction of the stimulated tentacle. A small CP is associated with the tentacle contraction. Similarly, threshold stimulation of the hydranth may result only in contraction of the hydranth region, with associated CPs, and in no apparent contraction of the stalk region. Responses of the polyp are more extensive and also more vigorous with increasing numbers and intensity of stimuli. The amplitude and number of recorded CPs decrement with distance from the stimulus site. Multiple CPs of large amplitude occur in regions of vigorous contraction, the number and amplitude diminishing in regions where contraction intensity is also reduced. During repetitive stimulation, contraction spreads to and involves a greater part of the polyp, an observation which appears to correlate with facilitation of CPs to each shock. Single threshold shocks generally give a localized polyp contraction and a single CP. Multiple shocks of threshold intensity or single suprathreshold stimulation of either proximal or distal regions of the polyp stalk can evoke multiple firing of the CP system. Some 30 or more CPs have been recorded to a single suprathreshold stimulus. Multiple firing of the CP system such as that shown in Figures 1D, E is correlated with a prolonged, continuous shortening of the polyp stalk, which often reduces the polyp to a stubby ball. In

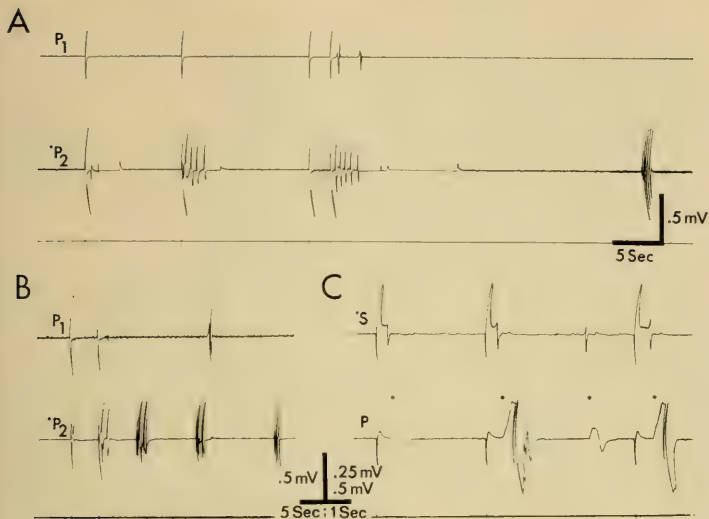


FIGURE 3. Interpolyp communication. A, simultaneous recordings from two interconnected polyps (P_1 and P_2) following application of four stimuli to one of them (asterisk). Largest events (stimulus artifacts) correlated to stimulus record (lower channel). Note that a burst (DBPs) occurs only in the stimulated polyp. B, same as A, but note that a CP occurs in P_1 shortly after a burst of DBPs in P_2 . C, simultaneous recordings from a polyp (P) and an attached stolon (S) while stimulating the same stolon (asterisk). Note the small potentials in S correlate with repetitive CPs (dots) in P which facilitate to each stimulus (marked in lower trace).

this condition, the polyp is refractory to further stimulation, and it may take 30 min or more before the polyp fully expands once again.

Dual recordings from a single polyp during multiple CP firing (Fig. 2) also show more vigorous contraction adjacent to the stimulus site and uniformly large CPs (Fig. 2B), while at a distant recording site the contraction may not be apparent initially but becomes increasing more vigorous throughout a CP burst. The CPs in such a burst show a marked facilitation (Fig. 2A). As a wave of contraction passes a recording site, subsequent stimulation can result in a marked reduction and even a defacilitation of CPs within a burst.

Stimulation of the hydranth region of a polyp, as shown in Figure 1B, can evoke both single and multiple CPs in addition to DBPs. In this record, the first of four stimuli initiated some four CPs and the third stimulus a single CP of smaller amplitude. And finally, some 12 sec after the fourth stimulus, the DBS fired a burst of potentials.

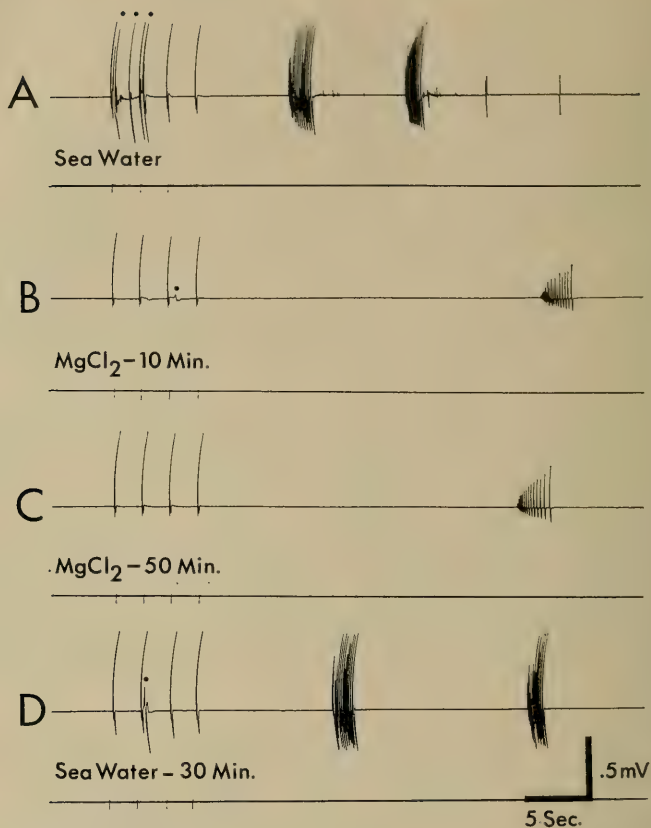


FIGURE 4. Effects of $MgCl_2$. A, recording from an isolated polyp in sea water. Stimuli in this and following records are marked in lower trace. B, same as A, after 10 min in $MgCl_2$. C, same as A, after 50 min in $MgCl_2$. D, same as C, following return of polyp to normal sea water for 30 min.

Interpoly communication

Communication from polyp to polyp by means of the interconnecting network of stolons has been difficult to demonstrate in wild colonies. In such colonies, the polyps arise from a tangled mass of stolons which makes it difficult to determine

which of the polyps are directly connected. In addition there are numerous symbionts (crustaceans, platyhelminths, protozoans) which live in association with the tangled stolons, many of which have been observed to feed on the soft tissues of the colony. It seems likely that these symbionts disrupt the structural organization of the colony, leaving whole portions of the colony or even individuals functionally isolated from each other. In only a few colonies did mechanical stimulation of one polyp affect the behavior of adjacent polyps. These were young colonies with new growth and no apparent damage to the stolons joining the two polyps. In all cases, very intense mechanical stimulation was necessary to show spread of excitation to an adjoining polyp, and in all cases only two polyps were involved.

Slide culture colonies provide a better preparation for the study of interpolyp communication. The stolons grow out in rather straight formations and give rise to polyps at intervals of about 3 to 5 mm. One can maintain cultures free of symbionts and also easily observe the integrity of the stolon network. Recording from and stimulating stolons and polyps which arise from them is greatly facilitated in culture colonies, and consequently it is easier to demonstrate interpolyp communication. Three examples of such are shown in Figure 3. In record A, recording electrodes were placed on two polyps in such a culture colony. One polyp, P_2 , was stimulated with four shocks in the region on the burst generator. CPs were recorded first in P_2 (the polyp stimulated), which eventually, after the fourth stimulus, were conducted through the stolon to P_1 where two small CPs were recorded. P_2 was observed to contract following the first stimulus, P_1 not until after the fourth stimulus. The DBS was activated in P_2 as can be seen by the delayed burst in this record, but the DBPs did not conduct through the stolons to the distant polyp (P_1). In Figure 3B, the polyp stimulated (P_2) gave rise to CPs and DBPs, the second burst of DBPs appears to activate the CP system of the distant polyp. While this is not conclusive evidence for an interaction between the DBS and CPS, it is supportive of additional evidence for such an interaction presented subsequently (Rushforth and Stokes, in preparation). There is no evidence that CPs initiate activity in the DBS.

In Figure 3C, recording electrodes were placed on a stolon (S) and a polyp (P) arising from that same stolon. The stolon was stimulated with three stimuli all of which initiated a small (150 μ V), fast spike in the stolon which correlated with CPs in the distant polyp. These CPs facilitated to each stimulus. In one case the small spike fired independently of an electrical stimulus, and it too correlated with a CP in the polyp and contraction of the polyp. DBPs were never initiated by stimulation of the stolon. Repetitive stimulation of a stolon results in a graded spread of excitation involving only the CP system. Spread of excitation to some 8 to 10 interconnected polyps in a colony has been observed, polyps closer to the stimulus site showing more CP activity than those more distant.

Effects of $MgCl_2$

The effects of isosmotic $MgCl_2$ on the mechanical and electrical activity of attached individual polyps are shown in Figure 4. Electrical activity and the visually monitored behavioral responses were first studied in normal sea water and when typically consistent responses were observed, isosmotic $MgCl_2$ was

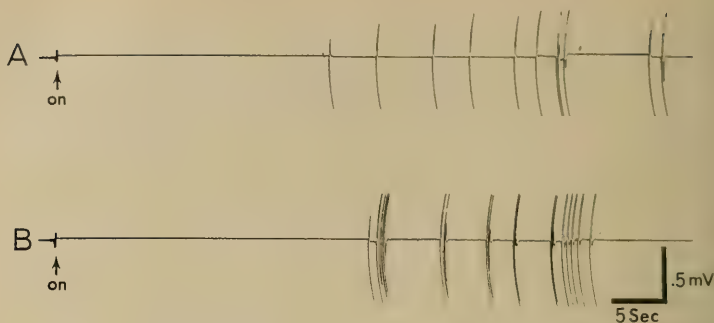


FIGURE 5. Light induced CP activity (A) and multiple CP activity (B) of *Clava* following a 15 min period of dark-adaptation. Onset of illumination is indicated by the arrows.

added to the experimental dish until a final concentration of 40% was obtained. The polyps were stimulated in the burst generator region with four shocks at 2-second intervals; the stimulus burst being repeated at 10-minute intervals. Only polyps giving both CPs and DBPs to two consecutive stimulus tests 10 minutes apart in normal sea water were used. A typical record in normal sea water is shown in Figure 4A. Both visual and electrical records were made following each stimulus regime. The results were similar for five polyps, one from each of five colonies.

Isosmotic MgCl_2 abolishes nearly all CP activity as well as the correlated behavioral responses after ten minutes exposure (Fig. 4B). The DBPs, however, persist in somewhat altered form and increased latency, despite the absence of a behavioral response. The bursts appear to consist of uniformly facilitating pulses of somewhat diminished amplitude from that in normal sea water. Delayed bursts have been recorded for up to 3 hours in MgCl_2 without further change. Both CPs and typical DBP bursts are restored following exposure to normal sea water for a brief period (10–30 min; Fig. 4D).

Effects of light

Whole colonies of *Clava* respond to sudden sharp increases in illumination after an apparent delay of some several seconds. Polyps contract symmetrically and often in distinct steps in much the same manner as occurs following CP activity. There does not appear to be a systematic co-ordination of polyp contraction throughout the colony; polyps nearest the source of illumination generally contract first, but there is no set order of responses. Synchronized tentacle depression characteristic of DBS activity has not been observed. The latency of the contraction responses coupled with the absence of tentacle depression led to a series of experiments designed to determine whether electrical events result in CP activity, DBS activity, or perhaps an as yet undescribed conducting system.

TABLE II

Responses of dark-adapted Clava polyps to the onset of illumination. means $\pm$ s.e. (N = 5).

	Number of pulses			Latency* to first pulse
	Before	During	After	
a) Intact polyp				
Midstalk	0.3 $\pm$ 0.2	10.9 $\pm$ 2.7	0.6 $\pm$ 0.3	35.4 $\pm$ 4.8
b) Intact polyp				
Hydranth	0.8 $\pm$ 0.8	13.5 $\pm$ 2.4	1.8 $\pm$ 1.8	23.4 $\pm$ 5.8
Prox. stalk	1.0 $\pm$ 0.7	12.1 $\pm$ 2.0	3.1 $\pm$ 2.7	24.9 $\pm$ 7.0
c) Transected polyp				
Hydranth	0.5 $\pm$ 0.5	6.3 $\pm$ 4.0	0.6 $\pm$ 0.6	10.2 $\pm$ 1.2
Prox. stalk	0.6 $\pm$ 0.2	11.8 $\pm$ 0.7	0.8 $\pm$ 0.4	30.4 $\pm$ 6.4

* Defined as the time interval in seconds from the onset of illumination to the initiation of electrical activity.

Recordings were made from single *Clava* polyps of a whole colony which had been dark-adapted for 15 min. The results of these experiments showed that polyps are quite sensitive to light, producing both single (Fig. 5A) and multiple (Fig. 5B) electrical pulses during the initial stages of stimulation. The numbers of pulses recorded from single (midstalk) and dual sites (hydranth and proximal stalk) were determined for a control period of 2 min before light stimulation, and a 2-min period following light stimulation. The light source for all experiments was a 6-V bulb from a microscope lamp set about 15 cm from the preparation. Light intensity was similar throughout. The experimental regime was repeated for a minimum of five trials with a 15-min period of dark-adaptation between successive light exposures, for five polyps. The results for single recordings for all experiments are shown in Table II. The mean number of pulses recorded during the 2-minute light exposures for all experiments were on the order of 10 to 40 times greater than either before or after the light stimulation period. The mean latencies (defined as the time interval from the onset of illumination to the onset of the electrical response) varied from about 10 sec in the isolated hydranth preparation to 35 sec in stalk recordings. Although most of the electrical activity appears in the form of single or doublets of pulses, they are often produced in distinct groups. The mean number of pulses per group is significantly different for the hydranth and proximal stalk regions (5.1 ± 0.8 and 2.0 ± 0.1 , respectively). An intermediate value of 3.4 ± 0.1 was obtained from midstalk recordings.

Electrical responses to the onset of illumination have been recorded also from isolated tentacles (three of five preparations) isolated hypostomes (three of five preparations) and hydranth preparations from which all tentacles and the hypostome were removed (six of six preparations). We have not examined isolated pieces of stolon.

Addition of $MgCl_2$ abolishes the characteristic responses to the onset of illumination within a period of five minutes. The only exception occurred in a single isolated hypostomal preparation which produced DBPs that persisted in somewhat modified form for three hr, with no observable behavioral correlate.

DISCUSSION

Evidence from this study indicates that the CP system of *Clava* is another example of a fundamental muscle activating conducting system found in hydrozoan polyps. Electrical potentials from this system are correlated with symmetrical contractions of whole, or regions of whole, polyps, and are similar in pulse characteristics and conduction velocity to the electrical potentials correlated with contractions in other hydroids. Such pulses have the common features of long duration (up to 500 msec), relatively large amplitude (up to 15 mV), and slow conduction velocity (2–21 cm/sec) when recorded externally (Rushforth and Stokes, 1978).

Activation of CPs in *Clava* results always in some degree of symmetrical shortening of the polyp in the direction of the interconnecting network of stolons. The polyp may shorten in one or more steps depending upon the intensity and frequency of stimulation. Following successive contractions, the polyp is reduced to a stubby ball. Furthermore, when contracted, the polyps are shielded between the branches or within the damaged flotation sacs of the alga upon which they naturally occur. These responses are adaptive in that they provide a limited degree of protection for the exposed, soft-bodied polyp from potentially hazardous environmental stimuli. Similar protective functions of polyp contraction are apparent also in *Hydractinia* where the polyps ultimately contract below a layer of chitinous spines (Stokes, 1974b); in *Millepora*, where the polyp contracts into a calcified skeleton (deKruif, 1976; and in *Obelia* where the polyp withdraws into a hydrotheca (Morin and Cooke, 1971a). Protective withdrawal, like the escape responses of insects and crustaceans with giant fiber systems, may be the major role of the CP system in *Clava*. Behavioral activities such as those associated with prey capture, feeding, and defecation are more complex and are probably integrated by other conducting systems within the polyp. Multiple conducting systems have been physiologically identified in all hydroid polyps thus far examined, though in no case is the behavior totally attributable to known conducting systems. *Clava* has at least two distinct conducting systems within the polyp. In addition to the CPS, a non-polarized Delayed Burst System (DBS) has been identified which produces programs consisting of bursts of pulses. Such programs are initiated after a long delay (about 20 sec) following stimulation. The DBS can be distinguished from the CPS by its somewhat higher threshold of activation; resistance to Mg^{2+} ; restricted location within the hydranth of the polyp; and different behavioral correlates (tentacle depression vs. polyp contraction). The potentials produced by the DBS also have different conduction velocity and pulse characteristics. DBPs have shorter duration (50–60 msec), shorter interpulse intervals (170–230 msec), and faster conduction (8–12 cm/sec) than multiple contraction pulses (cf. Table I).

Though the functional significance of the DBS is not known, there is evidence to suggest that it interacts with the CPS. Tentacle depression together with polyp contraction is observed during the initial delayed bursts following DBS activation. These initial delayed bursts often appear to contain CPs interspersed with the DBPs (Stokes and Rushforth, personal observations). The CP elements in the delayed bursts are absent in the latter phase of a long program of bursts.

when the polyp is reduced to a stubby ball and contractions are no longer apparent. In this contracted state the CP system is not evoked by electrical stimulation. In addition, exposure of a polyp to Mg^{2+} eliminates contractions, and results in delayed bursts which appear to consist of a single pulse type, presumably DBPs. These observations suggest that the CP system is excited initially by the DBS and polyp contraction is caused in the early phase of a burst program. However, the system becomes increasingly refractory and CPs drop out in the final stages of the program.

Simultaneous recordings from two polyps connected by a stolon support the hypothesis that the DBS excites the CPS. Activation of the DBS by stimulation of the hydranth of one polyp can trigger CPs in the same polyp which then give rise to a CP in a neighboring second polyp. Very small potentials recorded in the muscle-free stolons interconnecting the two polyps correlate with the observed polyp contractions. These pulses may represent activity in nerve cells of an interconnecting nerve net. Nerve cells have been identified in the stolons of *Hydractinia* (Stokes, 1974a) but have not yet been looked for in *Clava* stolons. Single or multiple CPs of one polyp can also serve to initiate CPS activity in a second interconnected polyp. However, we have observed no case of CP triggering of DBPs. Interactions of the DBS and CPS in individual polyps and interactions of the CPS from polyp to polyp provide for a means of colonial co-ordination. Föyn (1927) was able to identify members of individual, interspersed colonies of *Clava* by pinching one polyp and observing which additional polyps contracted. Co-ordinated responses of polyps comprising a colony have been observed also in *Cordylophora* (Josephson, 1961b) and *Hydractinia* (Josephson, 1961a; Stokes, 1974b) where they are presumed to be protective and co-ordinated by conducting systems underlying muscle contraction. Clearly all members of the colony would be served by advanced notice of a predator attempting to feed on one member of the colony.

The electrical activity evoked by dark-adapted *Clava* polyps which occurs after the onset of illumination has certain features of both DBS and CPS activation. The recorded latencies following the onset of illumination to the initiation of electrical activity are usually quite long, sometimes on the order of 35 sec. The latencies of DBPs evoked by electrical stimulation are often equally as long. However, despite such long latencies, the following evidence suggests that light activates the CPS; removal of the burst generator region by transection of a polyp well below the hydranth does not affect the generation of pulses in the remaining proximal stalk region, clearly demonstrating that the burst generator region is not necessary for the light induced responses. Though patterns of light-induced potentials sometimes consist of programs of burst, such bursts are closer in pulse characteristics to multiple CPs than DBPs. Frequently the light response consists of sets of widely spaced single or double pulses; Mg^{2+} abolishes the light-induced activity and the behavioral responses in the same time course as CPs.

The long latency of the photic responses in *Clava* is similar to that recorded for *Hydractinia* (21–70 sec; Stokes, 1972). Such long latencies may result from a similar mechanism of pulse generation and spread of excitation. In fact, it is a contraction pulse system (the SCP) which is activated by light in *Hydractinia*. The latency of the response may reflect the levels of photosensitive pigments which

have accumulated during the period of dark adaptation (Ballard, 1942). In *Clava* we have preliminary data showing that there is an inverse relationship between the length of the dark adaptation period and the latency to the response. The light receptors, be they photosensitive pigments or some as yet unidentified photoreceptor, would appear to be widespread throughout a polyp. However, since more pulses are induced in the hypostomal region, than in the body column and base of the polyp, there may be relatively more pigment or more photoreceptors in this region. In *Hydractinia* electrical activity occurs only by direct photic stimulation of the basal mat (Stokes, 1972). We have not examined the light sensitivity of *Clava* stolons.

Very little is known of the morphological substrates of the CPS or DBS in *Clava*. Preliminary histological studies utilizing reduced methylene blue show the presence of nerve cells in the stalk region. The burst generator region and the stolons remain to be examined. However, electrical recordings suggest that CPs are not solely a result of neuronal activity. The electrical potentials are too large and of too long in duration to originate from the small nerve cells. Furthermore, they are conducted much more slowly than one would expect for purely neuronal pathways. It has been suggested that similar large potentials from other hydroids are propagated in epithelial sheets via low resistance junctional specializations (Josephson, 1967). Septate junctions have been found connecting epitheliomuscular cells of *Hydra* (Wood, 1959) and *Hydractinia* (Stokes, 1974a) where they have been implicated in contraction responses. Josephson and Macklin (1967) have shown that the CP of *Hydra* is a transepithelial event. On the other hand, as Mackie (1970) suggests, conduction of such large potentials may combine both neuronal and epithelial elements.

In this study we have shown that the colonial hydroid, *Clava squamata* possesses a Contraction Pulse System whose properties are similar to the CP systems of other hydroids. It provides further evidence that the CP system is a common conducting system in hydroid polyps. Studies with other hydrozoans should indicate whether it is a universal feature.

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SUMMARY

1. At least two conducting systems are present in the colonial hydroid, *Clava squamata*, the contraction pulse system (CP system) which initiates symmetrical polyp contraction, and a delayed burst system (DBS) which is correlated with tentacle depression and polyp contraction.

2. The CP system has properties similar to contraction pulse systems of other hydroids; its electrical pulses are of large amplitude (greater than 0.5 mV) and long duration (150 msec), and slow conduction velocity (2–3 cm/sec).

3. The CP system courses through the polyps and their interconnecting stolons.

Electrical stimulation of a single polyp gives rise to CPs associated with contraction of that polyp, which sometimes can be recorded also in adjacent polyps.

4. Isosmotic $MgCl_2$ abolishes CPs and associated column contractions, but does not suppress delayed burst pulses.

5. Light initiates contractions of the polyp and correlated CPs.

6. It is postulated that the CP system of *Clava* is similar to contraction pulse systems previously described for other hydroids.

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AN ANALYSIS OF POPULATION STRUCTURE IN PACIFIC MOLE CRABS (*HIPPA PACIFICA* DANA)

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Hippa pacifica Dana, a hippid mole crab, inhabits the intertidal zone of tropical and subtropical Pacific island beaches. At Oahu, Hawaii, it primarily scavenges *Physalia*, the Portuguese man-of-war which washes onto beaches from the open ocean (Matthews, 1955; Wenner, 1977). At Enewetak Atoll, Marshall Islands, although it sometimes receives an abundance of *Physalia* (S. Smith, personal communication), the mole crab apparently depends more upon lagoon-produced mysids and other zooplankton which wash ashore at night (Wenner, 1977).

A comparison of population samples in this species, both in Hawaii and at Enewetak, provided some unexpected observations. In Hawaii, samples of *Hippa pacifica* suggested a remarkably consistent population structure. During a 5-month period, samples were essentially identical to one another, whether obtained from different beaches or from different parts of the same beach on Oahu. However, the consistent results obtained in Hawaii did not hold for extensive sampling at Enewetak Atoll. Samples from populations on different beaches at that atoll showed wide differences when sex ratio was analyzed as a function of crab size.

Wenner (1972) earlier had examined the question of sex ratio and size for many marine crustaceans. At that time he conjectured that the several distinct patterns found might be species-specific, on the basis of data available. However, this hypothesis failed with the initial comparison of data from Hawaiian and Enewetak mole crab samples. Hawaiian population samples fell into what had been termed an "intermediate" pattern, whereas the first year's samples at Enewetak formed the "reversal" pattern found in protandrous species such as the pandalid shrimps studied by Butler (see Wenner, 1972; Fig. 8).

The second year of sampling at Enewetak yielded conflicting data among various islets and led to a reconsideration of the premise that the sampling method provided representative data, even though this discrepancy among samples was not initially obviously related to sampling method (the same procedure had been used in all cases). To test the efficacy of sampling technique, a beach of limited extent was sampled at Enewetak in the usual manner, except that animals were not returned as had been done earlier. Instead, crabs were removed during a 3-week period until few or no crabs came to the individual bait stakes (sampling with removal). Initial samples, which represented the normal sampling procedure, could then be compared with the larger segment of population removed from the beach. The question became: In what ways did the initial samples represent the larger beach population?

MATERIALS AND METHODS

The study site at Enewetak Islet was a 250-m long beach which terminated on its eastern end at a solid-walled cargo pier and tapered in the other direction to

a narrow strip of sand among concrete blocks and limestone rubble. Much of the western half of the beach fronted a limestone reef flat. At low tides the sand on that portion of the beach bordered on exposed coral reef.

During a 3-week period in 18 separate sessions (1–2 hr each), a total of 4011 animals was removed from the beach with the use of a baiting procedure described fully by Wenner (1977). Stakes baited with shark meat and placed approximately 10 m apart along the entire stretch of beach during each sampling session attracted the crabs. Thirty to forty stakes were kept in the sand for approximately 20 min and were reset two or three times more during each session.

Mole crabs, apparently reacting to chemical stimuli (Matthews, 1955), scurried toward the bait as it was repeatedly covered by wave action. Successive sets of bait during each sampling session usually yielded progressively fewer animals per bait stake. By the end of each session, even fresh bait placed between the sampling stakes did not generate an increase in catch rate.

Animals were usually collected during the mid-point of an outgoing tide, because that appeared to be the optimal time (on the basis of earlier experience). They were occasionally collected during other parts of the tidal cycle in order to sample sand patches covered by water only at such times. Such attempts, however, never yielded as many animals per unit effort as did those run during outgoing tides. Accordingly, in some comparisons samples were grouped by twos in order to offset those small numbers and to better reveal trends.

After animals were sieved from the sand, they were measured with the aid of an automatic sizing device (Wenner, Fusaro, and Oaten, 1974). Tallies included size, sex, and percentage of females carrying eggs within each size class. Females were returned alive to beaches at the opposite end of the islet. Males were kept in a sea water table and eventually returned alive to the original beach as part of another experiment.

RESULTS

Changes in catch during removal

Animals, when considered by sex and size, did not come uniformly to the bait during the 3-week period. The overall catch of males fluctuated until mid-way through the program and then began tapering off (Fig. 1A). By contrast, female catch was greatest at first, with a rather consistent decline thereafter (Fig. 1B). Small females (those equivalent in size to the male size range—solid bars in Fig. 1B) showed much the same catch pattern as males, however, indicating that size rather than sex was the factor responsible for the differences in catch pattern between males and females.

One prominent feature of the change which occurred during the removal program was the selective catch of large females during the early part of that period (Figs. 1B, C). More than 80% of the 378 females caught in the first two samples were greater than 12.4-mm carapace length (the mean minimum size of egg-carrying females). In the last six samples, however, fewer than 18% of the females caught were at least that large ($N = 319$).

The percentage of those females larger than 12.4-mm carapace length which carried eggs also varied at the removal beach during the 3-week period (Fig. 1D).

In the first two samples combined, 64.2% of the 307 larger females carried eggs, whereas 85.0% of the remaining number of larger females had eggs ($N = 860$). A 9 by 2 χ^2 test for homogeneity failed ($\chi^2 = 77.00$, $P < 0.001$), indicating that the percentage of egg-carrying females changed significantly during the 3-week period. Since egg development time is approximately 20 days (unpublished results), such a change with time would not be unexpected.

Results from the other two islets which had been repeatedly sampled yielded somewhat more consistent data. At Jedrol (David) Islet, where the removal program was started later and run only 10 days, the percentages of large females in berry for the five samples were 87.2, 85.7, 86.0, and 83.6%, respectively, with data for the last two samples combined to eliminate small sample error ($N = 376, 434, 150$, and 116, respectively). Those percentages did not show a significant change with time ($\chi^2 = 1.05$, $P < 0.05$). At Boken Islet, two samples taken 6 days apart had 89.3 and 84.2% of the larger females in berry ($N = 337$ and 505, respectively), a marginal significant difference ($\chi^2 = 4.53$, $P < 0.05$).

Sex ratio fluctuated more markedly than any other variable measured during the removal program. The first three of the 18 samples yielded a total of 923 animals, with each of those three having a low percentage of males (27.7, 28.2, and 30.1%, respectively). In later samples the percentage of males varied widely, from a low of 40.6% males (fifth sample) to a high of 78.8% males (last sample). However, a persistent upward trend in that percentage became evident when the percentage of males was considered as a function of cumulative number of animals caught (Fig. 1E). Beginning with the fourth sample, the overall percentage of males increased uniformly. After 4011 animals had been collected, 48.4% of them were males. When those data were grouped into four blocks of 923, 1127, 896, and 1065 animals, a 4 by 2 χ^2 test yielded a value of 234.36 ($P < 0.001$). When the same test was run with the first group omitted, the data remained heterogeneous ($\chi^2 = 113.00$, $P < 0.001$). This level of significance, coupled with the close fit of the line to the points, indicates that males were under-represented in the first group of three samples and over-abundant in the last group of 1065 animals (last seven samples combined).

It was after the trend shown in Figure 1E began to emerge that a total of 1679 animals was removed from the beach on Jedrol (Rex) Islet, where a parallel set of results emerged (Fig. 1F). Unfortunately, time did not permit additional sampling at that islet.

Two samples from Boken (Irwin) Islet were also obtained. The male percentage there rose from 1.7 to 6.4% during removal of a total of 346 and 456 animals, respectively. Boken Islet differs from the other two in being downwind from the lagoon waters during trade wind conditions and is, presumably, in a more food-rich location (Fusaro, 1978b; Wenner, 1977).

Estimates of population size

Since catch per unit effort generally declined during removal trapping, and since rate of decline is directly related to size of total population and to number removed, the total population size can be estimated by various methods (Southwood, 1966, pp. 181-186). However, not all of the four conditions as listed by

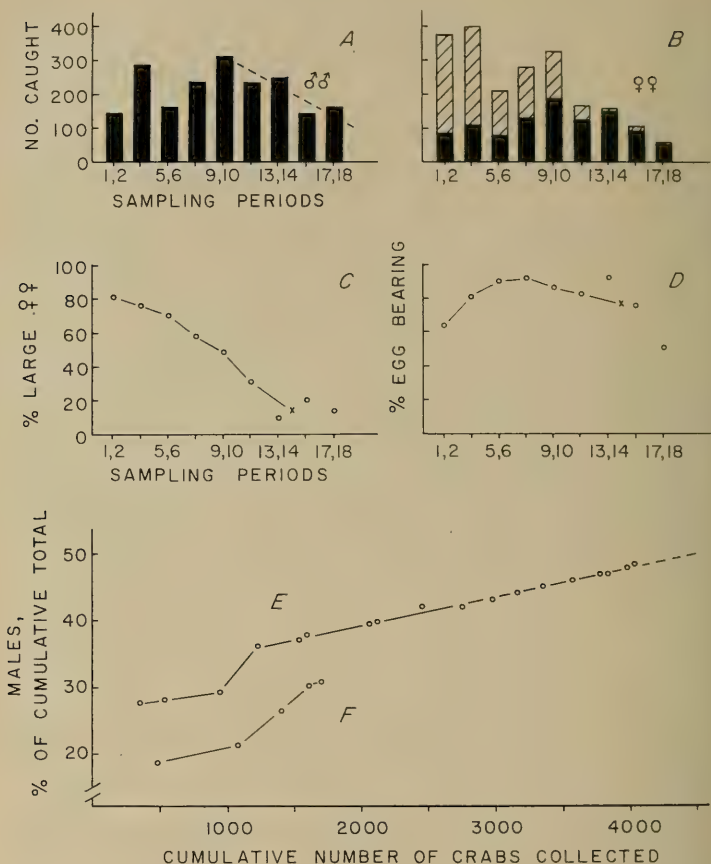


FIGURE 1. Changes in population samples during the three-week removal program. The bars depict sequential male (A) and female (B) catches, with the 18 samples grouped in pairs. Hours spent collecting each pair of samples were: 2, 2, 2, 2.25, 4, 2.5, 2.5, 3 and 3, respectively. The dashed line in Figure 1A indicates a consistent decline in catch per unit effort starting midway through the removal program; the solid portion of each bar in Figure 1B represents small females (females ≤ 12.5 -mm carapace length, the mean maximum size of males). The line in Figure 1C shows the decline in percentage of females which were large (females ≥ 12.4 -mm carapace length, the mean minimum size at onset of egg-bearing), while Figure 1D illustrates the differences obtained in egg-bearing percentages for those larger females during

TABLE I

Various estimates of the number of animals inhabiting the study beach on Enewetak Islet, determined by different methods based upon changes in catch pattern during removal sampling.

Method	Estimates of total population size			
	males	Total females	Small females	Total animals
Max. likelihood ($\pm$ s.e.)	9575 ($\pm$ 2996)	2374 ($\pm$ 35)	6091 ($\pm$ 6182)	6279 ($\pm$ 208)
Regression	8661	2532	4859	6539
"Corrected Regression"	2661	2389	1271	4658
Time-Unit	2967	2223	1518	4752
1:1 Sex ratio	2250	2250		4500
Total caught	1940	2071	1008	4011

Southwood (1966) were met in this present study. One condition in particular, "The chance of being caught must be equal for all animals" was clearly violated; males and small females were not caught as readily in the first eight sessions as were large females (first four bars in Figs. 1A, B). Nevertheless, a use of the various methods did provide a set of estimates (Table I), from which inferences can be drawn. In each case, estimates were made independently from the data for males, females, small females, and total number of animals, on the basis that the catch patterns differed markedly from one another in those different categories.

The method based on maximum likelihood, which Southwood (1966) called "the most accurate method", yielded the least consistent set of results. The male estimate (9575 ± 2995) exceeded the estimate for total number of animals (6279 ± 208). Likewise, the calculated number for small females exceeded the number estimated for all females.

Use of the regression method initially provided the same lack of consistency as that yielded by the maximum likelihood method; estimates for the number of males and small females were far too high to correspond with the estimates for total numbers of all animals or of all females. By inspection of Figures 1A and 1B, on the other hand, such a result would be expected, since males and small females clearly began a consistent decline in numbers only after the first eight samples (first four bars in those figures) had been removed from the beach.

Alternatively, one can adapt the regression method by applying it to the data

sequential removal. In both Figures 1C and 1D a weighted mean ($\bar{x}$) for the last three paired points compensates for small sample variation. The line in Figure 1E represents the percentage of males as a function of cumulative number of animals caught in the 18 samples at the main removal beach, while the line in Figure 1F shows the same relationship for animals taken from Jedrol Islet.

for the last 10 samples (last five bars). The estimates derived in each case can be added to the numbers caught in the first eight samples. Serious inconsistencies then disappear ("corrected regression" in Table I).

The "time-unit" method for estimating population size (Kono, in Southwood, 1966) relies heavily on data obtained at only three times (as well as on the cumulative catch at those times): the first sample, the mid-point sample, and the last sample. An application of that method to the data yielded estimates relatively close to those produced by the "corrected regression" method, with males and small females again being somewhat over-estimated because of their under-representation in the first sample.

Finally, if one assumes a 1:1 sex ratio for the population (the megalopa stage of *Emerita analoga* arrives on the beach in a 1:1 sex ratio, Wenner, 1972), an extrapolation of the line in Figure 1E to the 50% mark would yield an estimate of about 4500 animals. The 2250 females so estimated (assuming a 1:1 sex ratio) closely matches each of the other estimates for the total number of females.

The various estimates shown in Table I, qualified by the nature of the violation of conditions outlined by Southwood (1966), would indicate that most of the animals were removed from the study beach (between 87% and 93%, particularly if one relies on the quite consistent set of results for the total number of females).

The foregoing analysis now permits an assessment of how well the initial samples represented the larger beach population.

Modal size classes

The data for all samples combined fell into discrete modal size classes (Fig. 2), when separated by the method outlined by Cassie (1954). Data for males separated cleanly into only three modes, with the third mode having a slight inflection above the 95% level. The steep slope of Mode 3 suggests a maximum size for that sex. Female data actually fell into five modes. However, since the third and fourth modes did not differ appreciably, those data were combined and were thereafter treated as Mode 3 (Figs. 2, 3). The data thus reveal that the beach likely experienced four or five periods of recruitment during the year or two prior to sampling. (It should be noted, however, that the mode of largest females is based upon only seven animals caught during the first three sampling periods.)

To illustrate some of the differences in modal size class structure which arose during the removal process, data from the first three samples ($N = 923$) are herein compared to data obtained from the last six samples ($N = 876$). In all cases, modes were again separated by the same method.

Females in the first three samples ($N = 657$) fell primarily into the four or five modes mentioned earlier (Fig. 3A), but females in the last three samples ($N = 319$) provided data for only three distinct modes (Fig. 3B). The large displacement in the two sets of lines in Figure 3 does not represent an appreciable change in the size of animals within each mode. Rather, the displacement reflects a change in relative *percentage* of females which fell into each of the three modal size classes when one compares the beginning and end of the 3-week sampling period. For example, as can be seen in Figure 3, most females in the first three samples were in the third mode (79% of 657 females), whereas the second mode contained

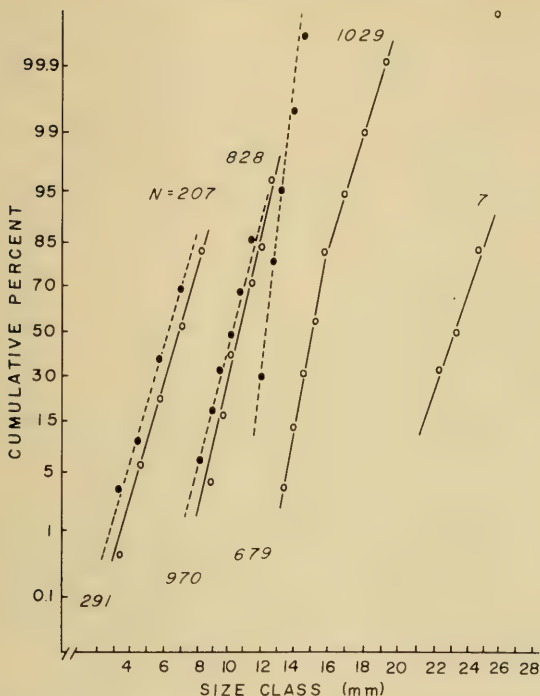


FIGURE 2. Male and female modal size classes for all data ($N=4011$). Males (broken lines) fell into three distinct size classes when modes were separated by the method of Cassie (1954). Females (solid lines) continued growth beyond the maximum male size. Approximate numbers within each mode are indicated below each line representing male modes and above each line representing female modes.

most of the females in the last six samples (72% of 319 females). Thus, two facets of modal size class structure should be distinguished: first, the estimated mean size of animals within each mode and second, the relative percentage of animals within each mode.

The data derived from the captured males (Fig. 4) differed from that for females in some important respects. Only two male modes occurred in the first three samples combined (Fig. 4A), while four or five female modes were evident in those combined samples (Fig. 3A). The smallest and largest females had no counterpart among the males in the first three samples. However, the last six

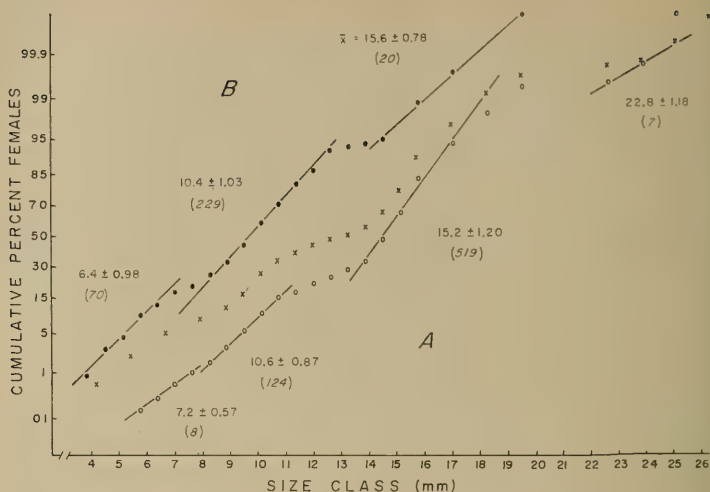


FIGURE 3. Changes in cumulative percentage of females as a function of size during removal. Data for the first three samples combined (open circles) fell into at least four modes (A). Data from the last six samples (closed circles) formed only three discrete modes, displaced upward on the graph due to a later catch of a higher percentage of smaller females (B). The x symbols indicate the relationship for all female size data ($N = 2071$). The sets of numbers below the lines indicate means, standard deviations, and approximate number (in parentheses) of animals within each mode in Figure 3A, and the numbers above the lines represent the same characteristics for Figure 3B. (See Fig. 2 for separation of modes for the total female data.)

samples yielded three modes for the males (Fig. 4B), modes which could be matched quite readily with the three modes representing smaller females in Figures 2 and 3B.

On the other hand, data for males obtained in the first three and in the last six samples (Fig. 4) did not show that same parallel relationship found in the female data (Fig. 3). The lines representing the upper mode for males in first and last samples converged. S. R. Haley (personal communication) and M. Page (personal communication) have concluded from laboratory results that males reach a maximum size and cease growth, though they continue molting. Females in the laboratory, according to Haley and Page, showed no such cessation of growth under similar circumstances.

The relative percentage of males which fell into each mode also differed markedly in the first and last samples (Fig. 4). The upper (third) mode in the first three samples contained approximately 75% of the 266 males, while 61% of the 557 males caught in the last six samples were in the second mode.

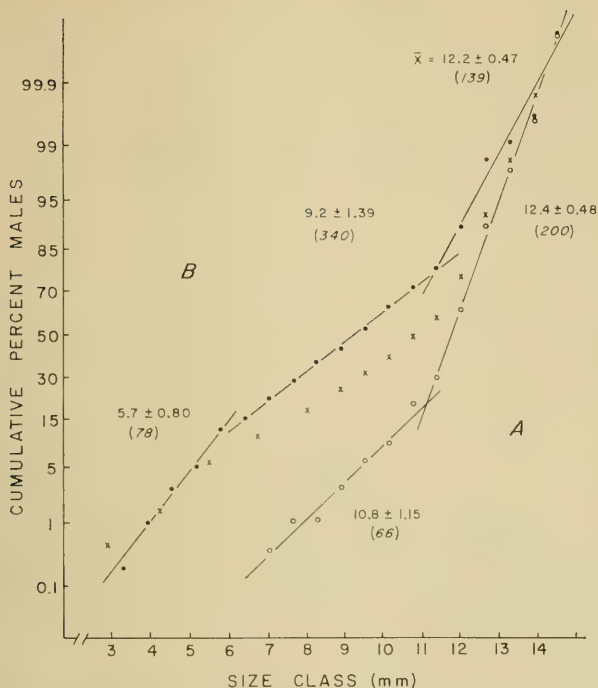


FIGURE 4. Changes which occurred in cumulative percentages of males, as in Figure 3 for females. Initial samples (A) yielded only two modes, as against the four to five modes for females. Final samples (B), however, resulted in three discrete modes for males. The convergence of the two lines representing the largest males agrees well with the concept of a maximum size reached by that sex. Means, standard deviations, and sample sizes are as shown in Figure 3.

Sex ratio curves

An earlier analysis (Wenner, 1972) revealed that sex ratio may vary with size in marine Crustacea. At the time, such variation was believed to form a pattern characteristic of a species or population. It is clear from more recent results obtained from mole crabs both in Hawaii and Enewetak that the "characteristic pattern" hypothesis is now untenable. The data published in 1972 indicated an "intermediate" sex ratio pattern for *Hippa pacifica* in Hawaii, but data gathered later at Enewetak in 1972 yielded a "reversal" sex ratio pattern for the same species (see Fig. 5A).

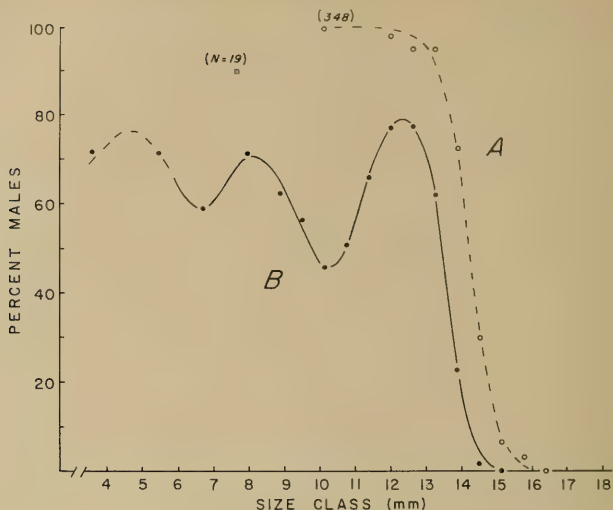


FIGURE 5. A discrepancy between sex ratio curves (sex ratio as a function of size—Wenner, 1972). A sigmoid curve ("reversal pattern") was obtained in 1972 when approximately 2000 animals were collected from six beaches on five different islets (A). A new pattern ("oscillation pattern") emerged when 4011 animals were removed from a single beach in the present study (B). Out of several hundred small animals in 1972, only one was female (the first point in Fig. 1A).

Data for initial samples in the current study yielded neither the Hawaii nor the earlier Enewetak sex ratio patterns; a sex ratio pattern existed which had not been found earlier (Fig. 5B). Instead, the percentage of males oscillated with increase in crab size. It is further evident that the number of oscillations corresponded well with the basic number of modal size classes found for males and females in the comparable size ranges (*i.e.*, Fig. 2).

The "oscillation" sex ratio pattern is what one might expect if the following conditions apply: first, the population consists of different cohorts which have arrived at different times from the plankton (three recent cohorts in this case), second, one sex grows faster than the other (females in this case—see Haley, 1979), and third, one sex reaches a maximum size, while the other sex continues growth beyond that size (females in this case).

For the first three samples of the present study, the sex ratio curve formed by the data for all animals greater than 10-mm carapace length nearly exactly matched that same portion of the curve for the total number of animals collected, as seen in Figure 5B. For the 86 animals in the first three samples which were 10 mm and smaller, however, only 28% were males, a point which falls far below

any of the points on the curve for all animals. This result corresponds with the under-representation of males as seen in Figure 4.

Despite the large discrepancy between sex ratio curves at the two localities and in different years, it should be noted that the sigmoid portions (for animals larger than 12 mm carapace length) of the curves in Figures 5A and 5B differ from one another by only about $\frac{1}{4}$ -mm carapace length at the 50% level in the graph. The mean maximum size of males (12.5-mm carapace length) also falls close to that same level. It is also noteworthy that an analysis of *Hippa cubensis* data (from Hanson, 1969) places that portion of the curve for his species between the two curves shown in Figure 5.

Size at onset of egg production

The mean minimum size of egg production can be a useful measure in population studies if one wishes to compare the success of animals which live in one habitat with the success of animals which live elsewhere (Wenner, Fusaro, and Oaten, 1974). It is essential, however, that initial sample data accurately represent the entire population before one makes that comparison. (In decapods the mean minimum size at which females can extrude eggs is one convenient measure of sexual maturity, provided conditions are optimum for egg production, since eggs are usually retained on the pleopods until they hatch.)

Unfortunately, samples of *Hippa pacifica* populations in both Hawaii and at Enewetak taken in earlier years did not permit a determination of the mean minimum size at which egg-bearing occurred because small females were seldom caught. The previous sections document one possible reason for such a failure—samples taken early in the sampling period did not include an accurate representation of small females present in that population.

The removal of a larger percentage of the population in the present study produced a sizeable number of smaller females and permitted the derivation of a curve which represented the mean minimum size of egg production for this particular beach ($\bar{x} = 12.4 \pm 1.02$ mm; $N = ca. 1000$). The curve had a striking similarity to the shape of a comparable curve published earlier for *Emerita analoga* (Wenner, Fusaro, and Oaten, 1974).

A question remained as to whether initial samples would indicate a different size at onset of egg production than would data for the population as a whole. Consequently, data for the first three samples combined were compared to the data for the total 3-week catch. Although the available sample size for the early data was quite small (and although more scatter existed among the points), it was evident that a marked difference did not exist between early samples ($\bar{x} = 12.7 \pm 4.64$ mm; $N = ca. 150$) and total data in this comparison. Only 0.3-mm carapace length difference existed between means for best-fit lines from all data compared to data for only the first three samples.

DISCUSSION

Pacific mole crabs (*Hippa pacifica*) apparently live only in the intertidal zone (Wenner, 1977); the same habit was reported for *Hippa cubensis* by Hanson

(1969). (However, see Borradaile, 1906.) This restricted habitat, together with a behavior of readily coming to bait, makes these animals particularly suitable for studies of crustacean biology. Large numbers of these carnivores can be collected in a relatively short time. In addition, the animals can be measured quite rapidly while still alive by means of a graded sieve (Wenner, Fusaro, and Oaten, 1974). In the present study, these combined attributes permitted measurement and removal of a large percentage of Pacific mole crabs from a beach limited in length. From population estimates (Table I), based on changes which occurred during sampling (Southwood, 1966), it would appear that approximately 90% of the animals which inhabited that short stretch of beach on Enewetak Islet, Enewetak Atoll had been removed.

Some of the contrasting results between first and last samples were unexpected, in the sense that earlier (1970-71) samples had yielded repeatable data. At that time samples from different beaches or islets were very similar, and repeated samples (sampling with replacement) from the same beach matched one another. In retrospect, one might conclude that the sampling bias could have been anticipated, since animals were caught by "trapping" (e.g., Gilbert, Gutierrez, Frazer, and Jones, 1976).

Trawling for animals can apparently lead to problems similar to those posed by trapping with bait. Gotshall (1972), while sampling shrimp during a 4-year period, experienced a bias problem similar to that encountered in the present study.

Small animals were caught less frequently at first and were primarily males: this discrepancy led to an imbalance in sex ratios throughout the sampling program. Hanson (1969; p. 15) earlier found such a discrepancy in his sampling program with *Hippa cubensis*. He wrote: "Length-frequency distributions of the (trapped animal) samples showed close conformation to those obtained by the more exact procedure of sieving sand samples from different levels of the beach, except for the smallest crabs (4-8-mm carapace length). Only 17.8% of the Bellair (trapped) samples were in this size range while 39.2% of the crabs from the Paynes Bay sieved samples were in this range." By contrast, Hanson derived an overall figure of 48.3% males for the animals from the several Paynes Bay beaches from initial samples, identical to the percentage we finally obtained only after removing more than 4000 animals from *one* beach. In addition, the first few samples in this present study were remarkably similar to one another, and by then 23% of the total catch had been removed. It was not until the fourth sample that a marked change in catch pattern occurred.

Although some changes during sampling were appreciable, the overall effort provided a rare opportunity: comparing all of the data from the removal beach with data obtained from the first few samples.

In studies of crustacean populations, a number of measures can assess just how discrete populations differ from one another in response to differences in environmental influences, but one must first have some confidence that initial samples have provided an accurate estimate of population characteristics before comparing populations with one another. Among the measures one can use are: first, modal size classes, including number of each sex in each size class and percentage of animals within each mode; second, size at onset of egg production (Wenner, Fusaro, and Oaten, 1974); third, extent of egg production, including

percentage of females carrying eggs and number of eggs per given size of female; fourth, "instantaneous" growth rate (Fusaro, 1978a), including field molt rate and size increment at molt; and fifth, sex ratio patterns (Wenner, 1972), including shape variations and pattern displacement. Age is generally not used, since that measure is very difficult or impossible to determine directly for crustaceans in nature (Wilder, 1953).

This current study has shown that initial samples provided reliable data for most of the above measures, but not all aspects, as discussed below.

Overall, the percentage of males and females within the different modes in the first three samples did not accurately represent the population structure obtained during the entire sampling program (Figs. 3, 4). Neither small males nor small females were properly represented in those first samples. Also, the seven large females caught at first were apparently the only very large animals on the beach.

However, although the percentage of males and females within each mode changed drastically during removal, the mean size of animals within each mode did not change appreciably (Figs. 3, 4). This means that population samples for this species can be compared to one another through time, if one takes into account the fact that the absolute percentages can vary greatly within each mode.

The first three samples yielded a reliable estimate for mean minimum size of egg production (Wenner, Fusaro, and Oaten, 1974), when compared to that estimate obtained in later samples or to that obtained from the total data (12.4-mm carapace length). Hanson (1969) found a 15-mm carapace length for the same characteristic in *Hippa cubensis*.

It is not yet certain whether the percentage of mature females which bears eggs is reliably determined from initial samples, but this seems to be the case. At the primary removal beach (Fig. 1D), an estimate derived from the first two samples (64.2%) was significantly lower than that obtained from all remaining samples (85.0%). However, data obtained from Jedrol Islet were quite consistent; the percentage of larger females (females greater than 12.4-mm carapace length) bearing eggs did not change during removal. It is therefore possible that an influx of food had occurred just prior to the beginning of sampling at the removal beach on Enewetak Islet (see Wenner, 1977, Table I). If so, it may be that the rising percentage of ovigerous females reflected that particular energy input.

At the removal beach, the sex ratio pattern (sex ratio as a function of size) obtained from initial samples quite clearly did not characterize the species or population as suggested earlier (Wenner, 1972, p. 344) for at least two reasons: first, small animals did not come to the bait in proportion to their numbers in the population when removal was begun; and second, it is now apparent from other work that both shape and position of the sex ratio curve can differ between populations and at different times of the year (see Fusaro, 1977).

A striking contrast between various Hawaii results (Wenner, 1972, Fig. 10; and Haley, 1979, Fig. 2) and Enewetak data (Figs. 5A, B, this study) can now be reconciled. The "intermediate" (Wenner, 1972, Fig. 10), "reversal" (Fig. 5A, this study), and "anomalous" (Haley, 1979, Fig. 2) sex ratio curves obtained from initial samples at various times likely did not accurately represent that aspect of population structure for the small animals. Rather, the differences between these patterns in the lower size classes probably reflect the degree to which

small animals had not been captured or the degree to which they might not have been seen during hand catching (Haley, 1979).

Three out of four of the previously described types of sex ratio patterns (Wenner, 1972) have thus now been obtained for a single species, apparently reflecting both sampling bias and changes in population structure. It is further apparent that each of those curves probably represented part of yet another sex ratio pattern (an "oscillation" pattern, Fig. 5B), a pattern which has now also been found for a confamilial mole crab, *Emerita analoga* (Fusaro, 1977). Furthermore, the number of oscillations in that pattern matches the number of major modal size classes found for males and small females (see Fig. 2), oscillations which may represent the number of major influxes of young in the recent months or years, modified by a differential growth rate between sexes (Haley, 1979).

In retrospect, it would appear that points which deviate from the sex ratio curve for other crustaceans (see Fig. 11 for *Calcinus latens* in an earlier analysis—Wenner, 1972) could well represent real deviations.

The results of this study indicate that males reach a maximum size in this species, a parameter which could be quite valuable for comparing populations one with another. In such comparisons, however, the largest male which can be found in each population is not the best estimate of that parameter (the "largest male size" normally increases with sample size). Rather, one can use the 50, 95, or 99% level in the mode representing the largest males. In Figure 2 the corresponding values would be: 12.5 ± 0.59 , 13.8, and 14.3, respectively. Statistical comparisons between populations are feasible, of course, only if one uses the first of these estimates (because one then has an estimate of standard deviation).

The initial three samples provided an accurate estimate of mean maximum size of males when compared to the data for all males. The first three samples combined yielded a mean and standard deviation of 12.4 ± 0.48 mm carapace length ($N = 200$); the remaining large males had a comparable mean of 12.5 ± 0.52 mm ($N = 479$).

The fact that males reach a maximum size while females continue growing beyond that size provides a related measure for comparing populations: an arbitrary point on the sigmoid portion of the sex ratio curve, where females first become dominant in the larger size classes (13.7 ± 0.47 mm at the 50% level in Fig. 5B). Since these sigmoid portions of the sex ratio curve are straight lines on probability paper, one can derive an estimate of the mean and standard deviation for that 50% transition point. This transition size was also quite accurately determined from initial samples. The first three samples yielded values of 14.0 ± 0.73 mm carapace length ($N = 393$), compared to the overall value shown above of 13.7 ± 0.47 mm ($N = 745$).

Southwood (1966) outlined methods by which some systems might be sampled so as to reduce the amount of bias inherent in estimates of population structure. Gilbert, Gutierrez, Frazer, and Jones (1976, p. 57) updated the approaches outlined and admonished: "The sample must represent the defined 'population' of animals or plants we wish to investigate." The concern of these workers has been supported only in part by the current study. Initial samples did accurately represent some aspects of population structure. It should also be stressed that researchers who would like to understand crustacean biology better are not merely "choosing suit-

able species for ecological work" (Gilbert, Gutierrez, Frazer, and Jones, 1976, pp. 58, 59). Marine forms can often be readily caught *only* by some form of trapping (*i.e.*, bait, nets, etc.). Although there is perhaps no way to know if initial samples contain a bias without going through a removal process, it is also obvious that a bias can be tolerated once one knows its nature.

Other researchers have reported similar discrepancies. In an extreme example, Bolin (1961) found that plankton hauls caught only female lantern fish (*Tarleton-beania crenularis*), while albacore tuna stomachs contained males but no females. The discrepancy became partially resolved when an unexplained event caused a massive death, resulting in millions of these fish being washed onto the beaches in central California. Of 521 specimens examined, 43.25% were males. Bolin speculated that males, being faster than females, could escape plankton nets. Conversely, he felt that females, having no photophores, would not be seen by tuna at that depth and could escape that mode of predation.

In a less extreme but still significant example, Gotshall (1972), who found marked discrepancies in catches of *Pandalus jordani* with respect to some year classes (size classes), suggested possibilities for those anomalous results, and reviewed some similar problems encountered by other researchers.

In the present study, although initial samples did not accurately portray a few of the conventional measures used by population biologists, reasonable estimates were gained of several parameters, as outlined above. The present study thus demonstrates the value of comparing data obtained from initial samples with actual population structure, especially when a biased sampling technique is the only feasible way to capture a sample from a population.

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SUMMARY

1. Essentially all of a population of mole crabs (*Hippa pacifica* Dana) was removed from an isolated beach at Enewetak Atoll, Marshall Islands. Estimates of population size, based on catch pattern, indicate that 87 to 93% of the animals were bait-trapped out of their restricted habitat. The sequential trapping permitted a number of comparisons between initial sample data and actual population structure.

2. The first three samples were large and yielded highly consistent data when compared to one another, but some aspects of population structure were nonetheless non-representative. For example, the percentage of crabs within each modal size class in the total population differed markedly from that estimated by initial samples. The location of those modal size classes did not change during the removal process.

3. After the first three samples (where male percentages were about 29%), sex ratio fluctuated wildly in individual samples, apparently as a consequence of size discrepancy in arrival at the bait. Eventually, males comprised about 48.4% of the 4011 animals removed.

4. Several other aspects of population structure were accurately estimated in early samples, including: mean size at onset of egg production, percentage of mature females carrying eggs, the sex ratio-size class relationship (for larger crabs), and the mean maximum size of males.

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SALT AND WATER BALANCE IN TWO MARINE SPIDER CRABS, *LIBINIA EMARGINATA* AND *PUGETTIA PRODUCTA*. I. URINE PRODUCTION AND MAGNESIUM REGULATION

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The excretory organs of decapod crustaceans function in magnesium regulation (Robertson, 1957; Lockwood, 1962; Potts and Parry, 1964) and to a lesser extent, in nitrogen excretion (Delaunay, 1931; Binns and Peterson, 1969). Magnesium regulation has been observed in decapods from a variety of habitats (Robertson, 1939, 1949, 1953; Webb, 1940), the usual pattern being a lowered blood concentration and an elevated urine concentration, with respect to the medium. This phenomenon has been investigated extensively in two osmoregulating crabs, *Pachygrapsus crassipes* (Prosser, Green and Chow, 1955; Gross and Marshall, 1960; Gross and Capen, 1966) and *Carcinus maenas* (Riegel and Lockwood, 1961; Lockwood and Riegel, 1969), but little is known about magnesium regulation in osmoconforming crabs.

A problem in the study of magnesium regulation, and other aspects of salt and water balance, is that of making accurate estimates of urine production rates. Cannulation is usually not feasible because of the geometry and delicacy of the excretory duct. Many methods have been used, perhaps the best known being nephropore-occlusion, but only a few estimates have been made for an extended period of time by the continuous collection of urine: *Procambarus clarkii* (Kame-moto and Ono, 1968; Ono and Kamemoto, 1969); *Paraneohrops zealandicus* (Wong and Freeman, 1976); *Cancer magister* (Holliday, 1977); *Callinectes sapidus* (Cameron and Batterton, 1978). A technique for the continuous collection of urine has been used in the present study.

MATERIALS AND METHODS

Specimens of the osmoconforming crab, *Pugettia producta*, were collected near the Bodega Marine Laboratory, Bodega Bay, California, and maintained at 10 to

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12° C, either in running sea water (SW) at the Bodega Marine Laboratory, or in filtered SW (about one crab per 4 liters) at the University of California, Berkeley, California. Intermolt crabs were used except where otherwise noted.

Estimates of urine production were made by continuous collection of urine. A polyester resin cast of the region surrounding the two nephropores was made in order to position two polyethylene tubes. In making the cast, the crab was clamped in a supine position and the pereopods secured with rubber bands. The ventral surface was dried with compressed air and those portions of the third maxillipeds distal to the ischia were cut off. The region surrounding the nephropores was lightly swabbed with vaseline which acted as a mold releasing agent. A transverse dam of plasticine, placed distal to the third maxillipeds, prevented the casting material from flowing into the mouth parts. A freshly mixed polyester resin, such as "G-R-R-I-P" (Idaho Chemical Industries, Inc.) was applied so that it extended posteriad to the plasticine dam, anteriad to the antennules, and laterad to about 5 mm beyond the nephropores. After 15 min, the cast was removed and the crab returned to SW. This procedure was carried out at least 24 hr before the start of an experiment.

The impressions of the opercula that cover the openings of the nephropores could clearly be seen in the cast. Each impression was used to center a hole of 0.063 inch (1.60 mm) diameter. A drill, larger by about 20% than the diameter of the opercula, was used to countersink the first pair of holes to a depth slightly greater than the diameter of the opercula. This allowed sufficient room for the opercula to open. Polyethylene tubing (P.E. Intramedic 190, Clay Adams) was pressed into the holes in the cast. Silicone grease was carefully applied around the periphery of the cast and between the two holes on its inner face. The crab was replaced in the clamp, the pereopods secured with rubber bands, and the region surrounding the nephropores dried with compressed air. The cast, with the attached tubing, was positioned and pressed onto the crab. In most cases it snapped into position and was firmly held in place by a rubber band.

The crab was then suspended in an aquarium so that the top of the carapace was just submerged. The seal between the cast and the animal was checked by blowing into the tubes and watching for air bubbles. The tubes were led over the lip of the aquarium, about 3 to 5 cm above the nephropores, and fed into two vials, positioned at about the same level as the nephropores. The urine was collected under mineral oil.

Urine production was also estimated by blocking the nephropores with Eastman 910 cement (Armstrong Cork Co.) and measuring the change in weight. The Eastman 910 was allowed to set for 10 min before the crabs were returned to SW. During this period, excess water was removed from the branchial chambers, the animals were dried for one minute with compressed air, and weighed.

The concentrations of sodium, magnesium, calcium, potassium, chloride, and ninhydrin positive substances (NPS), and osmotic pressure, in blood, urine and SW were measured. Blood samples were withdrawn by puncturing an arthrodiol membrane at a leg base with a drawn-out Pasteur pipet. Samples were centrifuged under mineral oil for 10 min in either an International Clinical Centrifuge at about 6000 rpm at room temperature or a Sorval model RC2-B, at 10,000 rpm at 1° C, the latter being used in the preparation of samples for osmotic pressure

determinations. Urine samples were also collected at the nephropore. A small hook, guided with the aid of a dissecting scope, was used to lift an operculum and the urine was collected in a drawn-out Pasteur pipet. Samples of plasma, urine and medium were stored under mineral oil in polystyrene vials.

Concentrations of cations were measured with a Perkin-Elmer model 290 atomic absorption spectrophotometer. Concentrations of chloride were measured on a Buchler-Cotlove chloridometer. NPS were determined by the method of Fowden (1951). Plasma was deproteinized by the addition of an equal volume of 10% trichloroacetic acid. For uniformity, urine received the same treatment. Samples were read on a Klett colorimeter against glycine standards. Measurements of osmotic pressure were made with an Advanced Instruments "Osmette" osmometer. Measurements of electrical potential difference across the body wall were made with an Analog Devices model 40J operational amplifier (input impedance, 10^{11} ohms) as a preamplifier and voltages were read on a Tektronix oscilloscope. Chlorided silver wires in 3 M KCl were used to make the electrical connections to 3 M KCl-agar bridges. One bridge served as the reference electrode while the other was placed in a hole in the top of the carapace, which remained out of water.

RESULTS

Urine production rates estimated by continuous collection

Figure 1 shows the results of an experiment where urine was collected over a 24-hr period. The sum of the cumulative volume of urine released from both nephropores has been arbitrarily fitted with a fifth order polynomial. The urine production rate, the first derivative of this polynomial, is also included. The rate of urine production was usually greater during the first 12-hr period than during subsequent collecting periods. The rates which will be reported were obtained after a 12-hr lapse from the start of collection. Urine release is intermittent, the interval between successive releases can vary greatly, and urine is usually released simultaneously from both nephropores.

Intermolt specimens of *Pugettia producta* (average weight, 101 g) in 100% SW produced urine at 6.40 ± 3.08 (22) % body weight (bw)/day, mean $\pm$ SD (N). Postmolt crabs (73 g) produced urine at 2.89 ± 3.68 (5) % bw/day and premolt crabs (65 g) produced urine at 29.5 ± 3.98 (3) % bw/day. A single classification analysis of variance indicates that there are significant differences among these means ($P < 0.01$) and *a priori* tests indicate that there are differences between the rates for intermolt and premolt crabs ($P < 0.01$), and intermolt and postmolt crabs ($P < 0.05$). The cause for these differences is not known.

Urine production rates for 10 crabs (114 g) were compared on two successive days. The average rate on the first day was $5.06 \pm 3.68\%$ bw/day; on the second day it was $5.48 \pm 3.27\%$ bw/day. These means are not significantly different (paired *t*-test). For one crab, urine production was measured for a 2-week period. During this time, the rate varied from 2 to 6% bw/day, the average being 3.02% bw/day. There was no apparent pattern to the daily fluctuations, and it is possible that the changes could be accounted for by changes in the volume of urine held in the bladders, which in *Pugettia* can exceed 5% bw (Cornell, 1976).

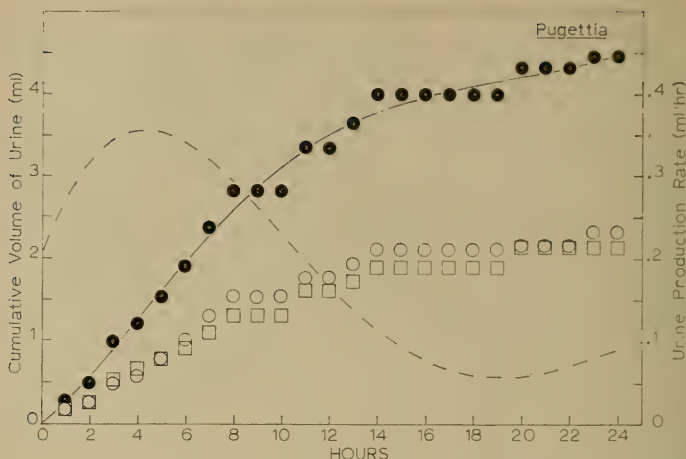


FIGURE 1. Urine production in a 46 g specimen of *Pugettia producta* from the time of placement of the polyester resin cast until the 24th hr. The cumulative volume of urine produced from the left and right nephropores is indicated by the square boxes and open circles, respectively, and can be read on the left ordinate. The sum of the cumulative volume of urine from both nephropores, indicated by solid circles, has been arbitrarily fitted with a fifth order polynomial, indicated by the solid curve. The first derivative of this polynomial, or urine production rate, is indicated by the dashed line and can be read on the right ordinate.

Urine production estimated from weight gain

Intermolt specimens of nephropore-occluded *Pugettia* (43.54 g) gained 3.38 ± 2.10 (12) % bw after 24 hr, while a control group (46.10 g) gained 0.43 ± 0.88 (9) % bw in the same period. Four crabs with blocked nephropores gained an additional 1.0% between 24 and 48 hr. These animals were markedly swollen, the posterior region of the carapace being lifted away from the abdomen. The urine production rate for intermolt crabs determined by weight gain is significantly less than that determined by continuous collection ($P < 0.005$, *t*-test), suggesting that the rates estimated by weight gain are under-estimates of the true rate, and that significant back pressures can occur.

Some major constituents of blood and urine

Measurements of some major constituents of blood, urine and medium for crabs in 100% SW are presented in Table I. An approximate statistical test (Sokal and Rohlf, 1969, p. 372), analogous to a single classification analysis of variance, was used to test for equality among means since these data are heteroscedastic.

The concentrations of sodium and chloride in the blood were both 98% of

their respective concentrations in the medium. In the urine, the concentration of chloride was about equal to, while sodium was 96% of, the concentration in the medium. The concentrations of magnesium, calcium and potassium in the blood were 88, 118, and 105% of their respective concentrations in the medium. In the urine, these same ions were 135, 124, and 114% of the medium, respectively. NPS were about 10 times more concentrated in the blood than in the urine. The osmotic pressure of the blood was about 2 mosM greater than that of the medium and the urine was isosmotic to the blood, but not statistically different from the medium.

The concentrations of inorganic ions were more variable in the urine than in the blood; in particular, magnesium was the most variable, as judged by the ratio of the standard deviation to the mean. Magnesium concentrations ranging from 48 to 128 mM were observed in the urine. Table II shows two correlation matrices, one each for the blood and the urine. In each matrix, a rank correlation coefficient has been computed for each inorganic ion with every other inorganic ion. There is a significant negative correlation between sodium and magnesium in the urine. There are also significant positive correlations between magnesium and calcium and between magnesium and potassium in the urine.

In the blood, magnesium concentrations were also relatively more variable than the other ions which were measured. A significant negative correlation between magnesium and calcium was found, the reverse of the condition in the urine. Significant positive correlations between magnesium and chloride, and between sodium and potassium, were also found.

TABLE I

Concentrations of some major constituents of the blood and urine of specimens of Pugettia producta in sea water. Relative concentrations are expressed as a percentage of the medium. Absolute concentrations of inorganic ions, ninhydrin positive substances (NPS) and osmotic pressure (OP) are expressed in mM, mM glycine, and mosM, respectively. F(df) indicates the F ratio of an approximate test with the calculated degrees of freedom. One and two asterisks denote $P < 0.05$ and $P < 0.01$, respectively.

Solute	Fluid Relative concentration Absolute concentration, mean $\pm$ sd _r (N)			F (df)
	Blood	Urine	Medium	
Cl ⁻	98.0% 524 $\pm$ 12.9 (13) 97.8%	99.4% 532 $\pm$ 27.9 (14) 96.1%	100% 535 $\pm$ 2.73 (6) 100%	4.24* (2, 18) 9.60**
Na ⁺	88.3% 450 $\pm$ 16.0 (23) 135%	135% 442 $\pm$ 22.1 (37) 124%	100% 460 $\pm$ 5.74 (6) 100%	75.7** (2, 30) 95.6**
Mg ²⁺	46.1 $\pm$ 2.48 (23) 118%	70.5 $\pm$ 19.2 (37) 124%	52.2 $\pm$ 0.65 (6) 100%	(2, 24) 95.6**
Ca ²⁺	11.8 $\pm$ 0.52 (13) 105%	12.4 $\pm$ 1.11 (22) 114%	10.0 $\pm$ 0.17 (6) 100%	(2, 43) 11.5**
K ⁺	10.2 $\pm$ 0.38 (13)	11.1 $\pm$ 1.13 (22)	9.74 $\pm$ 0.19 (6)	(2, 21) 38.4**
NPS	3.72 $\pm$ 2.00 (14) 100.2%	0.39 $\pm$ 0.12 (5) 100.2%	— 100%	(1, 13) 3.69*
OP	1016 $\pm$ 2.47 (10)	1016 $\pm$ 3.77 (10)	1014 $\pm$ 1.19 (10)	(2, 15)

TABLE III

Magnesium and sodium concentrations in the blood of normal and nephropore-occluded specimens of Pugettia producta in sea water. See text for statistical tests.

Ion	Time					
	0 hr		24 hr		48 hr	
	Normal	Occluded	Normal	Occluded	Normal	Occluded
Mg ²⁺ (mM) sd, N	47.1 2.05, 7	47.5 2.48, 10	46.8 1.01, 7	48.5 4.33, 10	46.1 2.44, 7	51.4 3.87, 10
Na ⁺ (mM) sd, N	448 14.8, 7	454 20.1, 10	450 4.5, 7	462 28.9, 10	446 16.8, 7	473 23.9, 10

The effects of nephropore-occlusion on magnesium regulation

Of the inorganic ions studied, magnesium represents the clearest example of an ion which is regulated by the excretory system. Its concentration in the blood, urine, and medium suggest that it is continuously diffusing down the concentration gradient from medium to blood, and that this gradient is maintained by its active removal by the excretory system. Estimates of the electrical potential difference across the body wall of three crabs indicate a difference of less than 0.1 mV. Thus, the driving force for the diffusion of magnesium from the medium to the blood must be the difference in chemical potential.

Blocking the nephropores should stop the removal of magnesium from the blood, and thus the magnesium level in the blood should rise, reaching equilibrium with the magnesium in the medium. The results of such an experiment appear in Table III. The magnesium concentration in the blood of the experimental animals with blocked nephropores increased; after 48 hr the ratio of the magnesium concentration in experimental crabs to that in control crabs was 1.12. There was also an increase in sodium concentration in the blood of experimental crabs, the above ratio being 1.06. This may indicate that sodium is also normally regulated below its equilibrium concentration. The sodium and magnesium data were separately analyzed by two-way analysis of variance after randomly removing the data for three crabs in the experimental group in order to facilitate the calculations. These analyses indicate that there are significant increases in sodium and magnesium concentrations in the blood of nephropore-occluded crabs ($P < 0.01$ for both ions in the sub-groups Normal and Occluded; for both ions, the F ratios were not significant in the sub-group Time, and for the interaction between sub-groups Normal and Occluded $\times$ Time).

In *Pachygrapsus crassipes*, Gross and Capen (1966) demonstrated that the magnesium concentration in the urine is a direct function of the time the urine is held in the bladder. This does not seem to be the case in *Pugettia*. When, after 86 hr, the magnesium concentration in the urine of nephropore-occluded and control crabs were compared, the magnesium concentration in the control crabs, 81.9 ± 26.8 (7) mM, was greater than that in the experimental crabs, 58.4 ± 9.93 (9) mM. This unexpected result seems to have been caused by chance, since the

concentration in the control group was greater than expected. Also, since one of the crabs in the experimental group had died, the remaining animals may not have been in good condition and the transport of magnesium could have been reduced. The experiment was repeated using a paired design and a shorter period of time to minimize the effects of individual variation and nephropore-occlusion. No differences were found in 10 crabs between the magnesium concentrations at 0 hr, 65.3 ± 20.7 mM, and 24 hr, 64.2 ± 18.3 mM, after nephropore-occlusion. These experiments suggest that the bladders of *Pugettia* do not secrete magnesium into the urine.

DISCUSSION

Decapod crustaceans have considerable ability to regulate their internal ionic compositions (Robertson, 1949, 1953). This appears to be independent of the ability to osmoregulate and has been defined by Robertson (1949, p. 182) as the "maintenance in a body fluid of concentrations of ions differing from those of a passive equilibrium with the external medium." Since the blood of decapods contains considerable amounts of non-diffusible, negatively charged protein, the internal/external concentration ratio of an ion can differ passively from unity. This ratio is expected to be less than 1.0 for the anions and greater than 1.0 for the cations. Robertson (1953) found, using a dialysis technique, that it was often 1.03 for the cations, except for calcium for which it was estimated that up to 20% was complexed with proteins. Greenaway (1976) has confirmed these results for calcium.

The present data suggest that *Pugettia producta* hypo-regulates magnesium and sodium; potassium is probably slightly hyper-regulated, while chloride is probably very close to its equilibrium concentration. From these data it is not possible to determine if calcium is regulated, since an unknown amount is complexed with proteins. The comparison of the concentrations of various ions in the blood of *Pugettia* with those of other decapod crustaceans, tabulated by Prosser (1973), indicates that the pattern of regulation in *Pugettia* is similar to that in other marine decapods.

Expressed as mM glycine, the blood and urine of *Pugettia* contain 3.7 and 0.39 mM NPS, respectively. It was the usual policy to use crabs which had not been fed for a week. Thus, NPS in the blood of *Pugettia* fresh from the field could be different. A comparison of these values with those reported for *Carcinus maenas* (Binns, 1969b; Evans, 1972) indicates that the blood concentrations are similar in both crabs when differences in technique are accounted for (see Evans, 1972). The concentration of NPS in the urine of *Pugettia* appears to be about half of that in *Carcinus*, but this can be quite variable and may not represent a true difference. The antennal gland of crustaceans is not a major route for nitrogen loss. Binns and Peterson (1969) estimate that in the spiny lobster *Jasus edwardsi*, 90% of all the soluble nitrogen is excreted extra-renally.

The urine production rate for intermolt specimens of *Pugettia*, determined by continuous collection, was 6.40% bw/day. However, the rate determined by weight gain was less than half of this, 2.95% bw/day after correction for the control value. There is good reason to believe that the former estimate is the more correct, the latter being reduced by back pressure. Although few data exist, there appears

TABLE IV

Magnesium excretion rates and magnesium permeabilities for some decapod crustaceans in sea water. One dagger indicates that C_m and C_b are magnesium concentrations (mM) in medium and blood, respectively. Two daggers: see text for discussion of the effects of the electrical potential on these values. Three daggers denote the references: (1) Gross and Marshall, 1960; (2) urine and blood $[Mg^{2+}]$ —Riegel and Lockwood, 1961; urine production rate—Binns, 1969a; (3) this report.

Animal (reference)†††	Blood Mg^{2+} mM	Urine Mg^{2+} mM	Medium Mg^{2+} mM	Urine flow rate % bw/day	Mg^{2+} excretion rate μ mol g·day	Mg^{2+} permeability μ mol ($C_m - C_b$)/g·day†
<i>Pachygrapsus</i> <i>crassipes</i> (1)	20	305	52.0	3.9	11.9	0.37††
<i>Carcinus</i> <i>maenas</i> (2)	31	250	52 (?)	4.4	11.0	0.52††
<i>Libinia</i> <i>emarginata</i> (3)	44	66	49.0	5.1	3.4	0.68
<i>Pugettia</i> <i>producta</i> (3)	46	70	52.2	6.4	4.5	0.72

to be a relationship between urine production and molt stage since premolt crabs produced urine at greater rates, and postmolt crabs produced urine at lesser rates, than intermolt crabs. The cause of this relationship is a matter for speculation.

The urine production rate was also determined by continuous collection for the osmoconforming spider crab *Libinia emarginata* (5.1% bw/day) and the freshwater crayfish *Pacifastacus leniusculus* (6.0% bw/day). At present, it is difficult to explain, considering water permeabilities and osmotic pressures of blood and media, why *Libinia*, *Pacifastacus* and *Pugettia* produce urine at comparable rates. Regardless, it is clear from many studies that most moderate-sized decapods produce urine at 2 to 10% bw day when tested at salinities representative of normal habitat salinity, the exception being the freshwater brachyurans which produce no more than about 1% bw day (Shaw, 1959; Thompson, 1970; Harris, 1975).

The present data suggest that the excretory system of *Pugettia* is responsible for the lowered magnesium concentration in the blood since magnesium is concentrated in the urine, and since blocking the nephropores results in elevated levels of magnesium in the blood. Calcium and potassium are also concentrated in the urine, although not to so great a degree as magnesium. There appears to be some relationship among magnesium, calcium, and potassium in the urine, the concentration of the latter two ions being positively correlated with that of magnesium. By contrast, the calcium concentration in the urine of *Carcinus* is independent of the magnesium concentration (Lockwood and Riegel, 1969).

The concentration of magnesium in the urine of *Pugettia* is negatively correlated with that of sodium. Similar relationships between sodium and magnesium have been reported for *Carcinus* (Webb, 1940; Riegel and Lockwood, 1961), *Pachygrapsus crassipes* (Prosser, Green and Chow, 1955; Gross and Marshall, 1960; Gross and Capen, 1966) and *Cancer magister* (Hunter and Rudy, 1975). Thus, some form of Na^+ , Mg^{2+} exchange mechanism may exist. Little is known about the mechanisms of magnesium transport. However, Holliday (1978) has found

that the net magnesium flux in the isolated bladder of *Cancer* is inhibited by ouabain and that only a small part of the opposing net sodium flux is associated with the magnesium flux. In the isolated gut of the insect *Hyalophora cecropia*, magnesium transport is independent of sodium and related, in a complex way, to potassium transport (Wood, Jungreis and Harvey, 1975).

The reabsorption of fluid from the urine, as suggested by the urine blood (U/B) ratio of filtration markers, is insufficient to account for the U/B ratios of magnesium in a number of decapods (Gross and Capen, 1966; Lockwood and Riegel, 1969; Franklin, Teinsongrusmee and Lockwood, 1978). In *Pugettia*, the U/B ratio of magnesium is about 1.5 and the U/B ratio of inulin may approach this value (Cornell, 1976). However, there is some difficulty in the interpretation of inulin U/B ratios in animals with large bladders (see Riegel, Lockwood, Norfolk, Bulleid and Taylor, 1974; and Cornell, 1976). Thus, it seems unwarranted to conclude that fluid reabsorption accounts for the concentration of magnesium in the urine of *Pugettia*.

Gross and Capen (1966) have shown that the magnesium concentration in the urine of *Pachygrapsus* is a function of the time the urine is held in the bladder. However, during nephropore-occlusion the magnesium concentration in the urine did not increase in *Pugettia*, suggesting that magnesium is concentrated in the antennal gland. A similar experiment on *Pachygrapsus* produced an increase in urine magnesium. In Figure 3 of their report, Gross and Capen (1966) presented results which suggest that the fluid entering the bladders contains 38 mM magnesium, about 20 mM higher than the blood. The urine of *Pugettia* is about 24 mM higher in magnesium than the blood. The implication is that the antennal glands of both animals perform equivalent tasks, and that it is the bladders of *Pachygrapsus* which concentrate most of the magnesium, a function which the bladders of *Pugettia* do not seem to perform.

The concentration of magnesium in the blood must be a function of at least three parameters: the permeability of the body wall, the excretion rate, and the electrochemical potential across the body wall. Neglecting for the moment the electrical potential, and dividing the weight-specific magnesium excretion rate by the concentration difference between the blood and the medium, an estimate of the permeability of the body wall can be obtained. In Table IV these quantities have been computed for four decapods. Taking into account the electrical potential does little to change these results. In *Pugettia* and *Libinia*, the potential is negligible, 0 ± 0.1 mV. Potentials of -2.0 mV (inside negative) for *Pachygrapsus* (Rudy, 1966) and *Carcinus* (Greenaway, 1976) have been reported for animals in SW. This is a small potential and for present purposes may be neglected, however, the permeability of *Pachygrapsus* and *Carcinus* may be slightly less than indicated. The data in Table IV suggest that those crabs which have much reduced the magnesium concentration in their blood have done so by lowering the magnesium permeability and by raising the rate of magnesium excretion.

The significance of magnesium regulation in crustaceans, first noted by Robertson (1949, 1953), is that a strong negative correlation exists between the activity of an animal and the magnesium concentration in its blood. Thus, animals with low magnesium levels tend to be highly active, while those with high levels

tend to be lethargic. Robertson (1953) has pointed out that high extracellular levels of magnesium have been found to block neuromuscular transmission in *Carcinus* (Katz, 1936) and to reduce the mechanical response of isolated crayfish legs to electrical stimulation (Waterman, 1941).

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SUMMARY

1. A new technique for the continuous collection of crab urine is described. Estimates of urine production, based on this technique, indicate that specimens of *Pugettia producta* in sea water produce urine at 6.4% body weight (bw)/day. Premolt and postmolt crabs produce 30 and 3.0% bw/day, respectively. Inter-molt specimens of *Libinia emarginata* produce 5% bw/day.

2. The urine production rate for specimens of *Pugettia*, estimated by weight gain following 24 hr of nephropore-occlusion, is 3.0% bw/day. This is significantly less than that determined by the continuous collection of urine, suggesting that back pressure can interfere with urine production.

3. Ion regulation was examined in specimens of *Pugettia*. When expressed as a percentage of their concentrations in sea water, the values in blood plasma of chloride, sodium, magnesium, calcium, and potassium are 98, 98, 88, 118, and 105%, respectively, for crabs in sea water. Likewise in the urine, the values for these same ions are 99, 96, 135, 124, and 114%, respectively. Ninhydrin positive substances, measured with glycine standards, are 3.7 and 0.39 mM in blood plasma, respectively. The electrical potential across the body wall of both species of crab is zero.

4. In *Pugettia*, blocking the nephropores causes an increase in the magnesium concentration in the blood, suggesting that the excretory system is mainly responsible for regulating this ion. However, blocking the nephropores causes no change in the magnesium concentration of urine stored in the bladder, which suggests that the antennal gland is mainly responsible for concentrating magnesium in the urine. *Libinia* and *Pugettia* excrete magnesium at a lower rate, and are more permeable to magnesium, than brachyurans which are strong magnesium regulators.

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DIETARY FATTY ACID AND TEMPERATURE EFFECTS
ON THE PRODUCTIVITY OF THE CLADOCERAN,
MOINA MACROCOPA

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Analyses of the relationships between physical parameters and the quantity and quality of food in relation to zooplankton physiological processes such as ingestion, assimilation (growth) and reproduction have been scarce. These studies are difficult since experimental techniques have lacked means to keep biotic factors constant while modifying a physical factor, or vice versa. As a result, the nutritional quality of a particular food under certain physical conditions has not been evaluated.

The study of interactions between the quantity and quality of food available for a zooplankter and existing physical parameters is very important. The structure of zooplankton communities may be partially determined by the relative abilities of fauna to efficiently utilize and process the available food and thereby satisfy their individual nutritional requirements.

Investigators who have classified the nutritional suitability of a particular food for a predator have observed effects on ingestion, assimilation and reproduction. No study has adequately controlled experimental conditions so that the interrelationships among a physical factor, the chemical composition of diet, and the population dynamics of a zooplankton species could be identified.

Stuart, McPherson, and Cooper (1931) studied the relative value of a variety of bacterial species as food for the aseptic cladoceran, *Moina macrocopa*. They found differences in growth, fertility, and fecundity. Lefevre (1942) demonstrated that the normal growth and reproduction of various cladoceran species were dependent upon the suitability (physical or physiological) of species of fresh water algae. Monoxenic culture of two species of Crustacea, *Artemia salina*, and *Tigriopus japonicus*, an harpacticoid copepod, by Provasoli, Shiraishi and Lance (1959) demonstrated that many unialgal diets either failed to permit growth to adulthood or allowed only a few consecutive generations. Interestingly, a phytoplankton species that was nutritionally good for one species was not always good for the other species. The apparent nutritional deficiencies of some of the unialgal diets could often be rectified by the addition of specific vitamins or other organic compounds (Shiraishi and Provasoli, 1959). Lee, Tietjen, and Garrison (1976) observed a seasonal "switching" of nutritional requirements for *Nitocra typica*, an harpacticoid copepod from salt marsh aufwuchs communities. In these studies the comparative nutritional value of unialgal diets of various species and strains was determined by growth rate and fecundity measurements. In some cases the nutritional adequacy of a particular algal diet was temperature dependent. Guerin and Gaudy (1977) and Gaudy and Guerin (1977) grew the harpacticoid copepod, *Tisbe holothuriae*, on a variety of artificial chemically undefined particulate diets. The dry weight, elementary chemical composition, fecundity, sex ratio, and result-

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ing population dynamics of this species were significantly affected by the quality of food. Schindler (1971) showed that the assimilation efficiencies of the copepods *Diaptomus gracilis*, *Cyclops strennus*, and the cladoceran, *Daphnia longispina* are dependent upon the type of food eaten. Roman (1978) speculated that the successful association of the blue-green alga *Trichodesmium* and the harpacticoid copepod *Macrosetella gracilis* may be due to the copepod's efficient conversion of the carbon and nitrogen fixed by the alga into secondary production.

A precise understanding of how zooplankton population dynamics are influenced by the chemical composition of the diet can only be realized when proper conditions will permit the experimenter to independently control qualitative and quantitative changes. The axenic culture of a zooplankton on a chemically defined artificial diet would satisfy such controlled conditions. Such a diet must permit not only adequate ingestion, digestion, and assimilation but also continuous reproduction so that large populations and successive generations may be studied.

The biphasic medium for the axenic culture and continuous reproduction of *Moina macrocopa* formulated by Conklin and Provasoli (1978) provides for the precise study of food/zooplankton relationships. The medium consists of a soluble phase and a particulate phase. Water soluble vitamins, nucleic acids, amino acids, and inorganic salts comprise the soluble phase, while coagulated protein-starch and protein-lipid particles comprise the particulate phase. The medium has permitted the first analysis of the nutritional requirements of a crustacean (Conklin and Provasoli, 1977). By employing this medium the experimenter can control the chemical composition of the diet as well as the mode of presentation to the herbivore (i.e., number of particles/ml, amount of chemical compound/particle). Since the particles, unlike natural phytoplankton and bacteria, are inert to the chemical or quantitative changes associated with physical environmental factors, precise relationships between diet composition and some physical variable can be determined.

This study is directed toward analysis of dietary fatty acid and temperature interrelationships and the effects upon the productivity of *Moina macrocopa*. Undoubtedly there are other chemical compounds which affect the normal growth and reproduction of *Moina* (Conklin and Provasoli, 1977). Experimentation with fatty acids was chosen because, for this class of compounds, there are unique compositional differences amongst the orders of algae (Wood, 1974) and because past research demonstrates the essentiality of lipid factors for sustained fertility in *Moina macrocopa* and *Daphnia magna* (Provasoli, Conklin, and D'Agostino, 1970; Viehoveer and Cohen, 1938).

The importance of dietary lipids for the Crustacea was also inferred from the research on the phylogenetically related insects; lipids affect their metamorphosis, diapause, fertility, and fecundity (Beck, Lilly, and Stauffer, 1949; Vanderzant, Kerue, and Reiser, 1957; Tamaki, 1961; Dadd, 1960; Nayar, 1964; Adkisson, Bell and Wells, 1963; Bull and Adkisson, 1960; Foster and Crowder, 1976; Ikan, Stanic, Cohen and Shulov, 1970; Chumakova, 1962).

MATERIALS AND METHODS

The organism employed in this research was *Moina macrocopa americana*, a member of the order Cladocera. Descriptions and illustrations may be found in

Goulden's monograph of the Moinidae (Goulden, 1968). Reproduction occurs primarily by parthenogenesis. At times sexual females and males will appear in populations and sexual reproduction will occur (D'Abramo, in preparation). Parthenogenetic eggs are released from a pair of ovaries into a brood pouch. The neonates are born viviparously and are miniature images of the adults. Neonates pass through four stages of growth (instars) before becoming sexually mature. During the fourth or adult instar a female lays her first brood of eggs. Generation time is temperature-dependent but is rapid compared to many other Crustacea (4-5 days at 26° C).

Axenization

A serial dilution method as described by Conklin and Provasoli (1978) was employed to free the experimental organisms from bacteria. Recently born neonates were washed for 15 min in 15 separate baths composed of 10 ml of DM₇ medium (Provasoli and D'Agostino, 1970) in 6-cm diameter Petri dishes. All baths contained two drops of an antibiotic mix (D'Agostino and Provasoli, 1970). The eighth bath of the series contained a suspension of bacteria-free *Chlamydomonas reinhardtii* (GMS⁻), to allow for feeding and clearing the gut of bacterial flora. After the entire washing procedure, individual animals were transferred to 20 × 125 mm screwcap test tubes containing 10 ml of DM₇ and a suspension of *C. reinhardtii*. After two days growth at room temperature under a bank of cool white fluorescent bulbs, 0.5 ml of the culture medium was dispensed into semisolid DA medium (D'Agostino and Provasoli, 1970) for detection of any bacterial or fungal contaminants. This sterility test was incubated at room temperature for 15 to 20 days.

Once monoxenic cultures were established and found aseptic, young were aseptically transferred to 20 × 125 mm screwcap test tubes that contained 10 ml of a sterile artificial medium, K₇33, which is a modification of the artificial medium developed for *Moina* by Conklin and Provasoli (1977) (Table I). Acclimation to the artificial medium occurred within three generations and was determined by visual observation of the number of progeny per brood and of a normal swimming behavior. Culturing of animals fed artificial food was done in the darkness at room temperature. Growth in the dark discourages algal reproduction and all traces of algal cells generally disappeared by the third transfer in artificial media.

Populations of *Moina* were maintained on artificial and natural diets. Maintenance populations on artificial medium K₇33 were incubated at 19° C in darkness to prevent photodeterioration of riboflavin. Growth at this temperature slowed population growth rates, thereby increasing the time between transfers. Populations fed *Scenedesmus naegalii* (Chodat) and *C. reinhardtii* (GMS⁻) were grown at 19° C with a 16:8 L/D photoperiod. New cultures of natural and artificial diets were usually started every three weeks.

Diet formulation

The artificial media used in the experimentation with *Moina macrocopa* were modifications of the F1 medium of Conklin and Provasoli (1977). Modifications may be found in Table I. The media contained two types of particles, starch-

TABLE I
Modifications of Conklin-Provasoli F1 Medium.

Common basal medium (per cent w or v/v)

- Changes: (a) Metal mix P II to metal mix L; Metal mix L, 1 ml = $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, 3.81 g; $\text{Zn}(\text{as } \text{SO}_4^-)$, 0.30 mg; B, 0.12 mg; $\text{Mn}(\text{as } \text{Cl}^-)$, 0.087 mg; $\text{Fe}(\text{as } \text{NH}_4\text{SO}_4^-)$, 0.06 mg; $\text{Co}(\text{as } \text{Cl}^-)$, 0.024 mg; $\text{Cu}(\text{as } \text{SO}_4^-)$, 0.024 mg; $\text{Mo}(\text{as } \text{NH}_4^+)$, 0.036 mg.
 (b) Liver infusion L25 at 70 mg to defatted liver infusion L25 (lipid extraction by chloroform/methanol (2/1) (v/v) for three hr under N_2) at 40 mg.

- Additions: (a) glycogen 10 mg
 (b) globulin + 2X crystalline egg albumin, 1 ml, 1 ml = bovine α globulin (Fraction IV) 2.25 mg + 2X crystalline egg albumin (ICN), 0.75 mg. Mixture is formed by dissolving components in water; coagulating this mix by autoclaving; homogenizing (2600 rpm for 5 min) the coagulum to produce particles; autoclaving the particles and rehomogenizing.

- Deletions: (a) DF_2

Particles

- Changes: (a) SA gel particles: 1 ml = 10 mg rice starch + 4.10 mg 2X crystalline egg albumin.
 (b) FV particles: 1 ml = 8 mg 2X crystalline egg albumin + 0.75 egg lecithin + 1 mg BHT (butylated hydroxytoluene) + 0.66 ergocalciferol + 0.25 retinopalmitate + 2 mg dl- α -tocopherol + fatty acids in variable quantities and qualities, depending upon experiment. Fatty acids for K₇₃₃ medium: 1 mg palmitic acid + 0.3 mg oleic acid + 0.7 mg linoleic acid + 1 mg α -linolenic acid.

Artificial medium

Basal medium 96 ml + 3 ml SA gel + 1 ml FV particles, pH = 8.0, Particle concentration = 540×10^3 ml

protein and lipid-protein. All inorganic and organic additions were made from prepared stock solutions. Generally inorganic stock solutions were stored at room temperature while organic solutions or suspensions were stored either at 8° C or frozen. To prevent bacterial contamination, 0.5 ml of a volatile preservative solution (Hutner and Bjerknes, 1948) was added to all stock solutions. The volatile preservative vaporizes during autoclaving. Stock solutions were usually renewed within a four-month period. All fatty acids used in the diets were 99+% pure and were stored frozen under a N_2 atmosphere. Prepared media varied in the qualitative and quantitative composition of fatty acids absorbed onto the protein particles. Qualitative fatty acid additions were made to simulate the unique proportional differences found amongst four orders of algae, Cyanophyceae, Cryptophyceae, Chlorophyceae, and Bacillariophyceae (Table II).

Since the work involved the effect of quantitative and qualitative changes in dietary fatty acids upon the productivity of *Moina*, concern developed regarding the possible differential uptake of particular fatty acids by the albumin. To insure that results could be genuinely attributed to particular diets, particles prepared 2 months previously were subjected to a lipid compositional analysis by thin layer and gas chromatographic techniques. These analyses were kindly performed by Dr. David H. Beach of the Department of Microbiology, State University New York, Upstate Medical Center at Syracuse. Lipid analysis of the particles revealed

TABLE II

Fatty acid mixtures simulating average percent composition of representative algal classes (total fatty acids = 3 mg).

Fatty acids (mg)	Mixtures			
	Cyanophyceae ¹	Chlorophyceae	Cryptophyceae	Bacillariophyceae
14:0 (myristic)	—	—	0.30	—
16:0 (palmitic)	1.25	1.00	0.45	0.75
16:1 (palmitoleic)	0.80	0.10	—	1.20
18:0 (stearic)	0.12	—	—	—
18:1 ω 9 (oleic)	0.30	0.25	0.30	0.15
18:2 ω 6 (linoleic)	0.40	0.35	0.30	—
18:3 ω 3 (α -linolenic)	0.13	1.30	0.90	—
20:5 ω 3 (eicosapentaenoic)	—	—	0.45	0.90
22:6 ω 3 (docosahexaenoic)	—	—	0.30	—

¹ The 18:4 (octadecatetraenoic acid) which comprises from 15 to 30% of the cryptophyceae fatty acids was not incorporated into the mixture because of the lack of a conveniently available and highly pure (99 + %) source.

that the experimentally intended dietary differences, both qualitative and quantitative, were genuine. The lipophilic albumin had no tendencies toward differential absorption of the fats and vitamins. Uptake was complete.

Determination of particle size and particle concentration—optical transmission relationships

The number of particles in media of different particle concentrations was determined through the use of a Coulter Counter (Model Z_B, Counter Electronics, Hialeah, Florida). The counter was calibrated with the use of 10.2- μ diameter pollen. Counts of artificial particles were performed using a 100- μ aperture, an amplification of four and an aperture current equal to one half. The lower and upper threshold settings were 1 and 40, respectively. The threshold factor measured as the average volume of the known system divided by the lower threshold dial setting at half count equalled 16.8186. Particle size frequency distribution for a sample was determined using a Coulter Channelyzer (base channel threshold = 1, window width = 100) and an X-Y Recorder II for automatic plotting. The size of the particles was determined by employing the formula, Channel number $\times$ window width/100 + Base Channel Threshold $\times$ Threshold Factor = cubic microns.

Aliquots of variable volumes from samples of percent transmissions ranging from 45 to 85% were diluted to 20 ml by means of a special electrolyte solution, Isoton II (Curtin Matheson Scientific, Inc.). From these diluted suspensions ten separate 500- μ l samples were counted, from which an average was computed. Counts for these samples ranged from 10,000 to 30,000 per 500 μ l. Background counts did not exceed 100 per 500 μ l. The derived average particle concentration (number per 500 μ l) was then multiplied by the dilution factors to obtain the number of particles per ml. A standard curve relating particle concentration (number/ml) to per cent transmission was then constructed. Particle concentration within the media was altered by additions from the stock mixtures. All prepared media were

adjusted to pH 8.0 with a pH meter and were then dispensed with a macro-pipette (Macroset-Oxford Laboratories) as 10 ml aliquots into 20 × 125-mm screw cap culture tubes (Pyrex # 9825). The media in the culture tubes were then autoclaved, and, after cooling to room temperature, were stored at 8° C until use. Experiments involving the comparative quality of diets were all performed with media containing the same initial number of particles. Normal concentration was 540×10^3 particles/ml.

Preparation of inoculum and productivity determinations

To determine the relative nutritive quality of diets with each particular fatty acid composition, 10 to 12 first instar females were inoculated into separate culture tubes containing the same variable. These inocula were neonates of the first and second broods of single females that had been previously isolated from maintenance cultures and grown at 19° C in 20 × 125-mm screw cap culture tubes containing 10 ml of K₇33 media. From these, replicate inocula populations were allowed to develop by parthenogenesis at various temperatures and defined times. The particles of the artificial media were kept in suspension by daily mixing with a Vortex-genie mixer (Scientific Industries, Inc.). Particle sedimentation rates were slow and most of the particles (*ca* 70%) would remain in suspension over a 24-hr period. From the 10 to 12 growing populations a set of three or four culture tubes were harvested at three different predetermined particle concentrations (60–70%, 70–80%, 80%+ optical transmission at 650 nm). Optical transmission was measured by an instrument similar to a Spectronic 20 (Bausch and Lomb) but modified to accept culture tubes.

The sequential harvesting at different particle concentrations permitted an analysis of the changing structure of the *Moina* populations through time. Animals were killed by the addition of 0.5 ml of ethyl alcohol and then were transferred by pipette to a modified Bogorov counting tray (Wickstead, 1965). With the aid of a binocular stereoscope (36×) each individual comprising a population was counted, sized, and categorized. Categories included female instar I–IV, male instar I–IV, adult females, adult males, ephippial females, and ephippia. Measurements of the animals were made with the use of an ocular micrometer. Measurements were made anteroposteriorly, from a point just distal to the eye to the caudal tip of the carapace.

Average dry weights of the four female instars were determined by selecting a sufficient number of animals of a particular instar and placing them on a pre-tared aluminum dish. These samples were then dried at 60° C for 24 hr in a laboratory oven (Model 10–200C, Grieve-Hendry, Co., Chicago, Ill.), cooled for one hr in a desiccator, and weighed immediately on a Cahn gram electrobalance (Model G-Cahn Instrument Co., Paramount, California). Additional weighings of a sample were performed until no change in weight could be detected. Average dry weight of a particular instar was determined by dividing the biomass in the dish by the number of instars which comprised the sample. Three samples of well-fed animals were taken for the average weight determination of each instar. Each sample was derived from a population growing on a different diet. A $\log_{10}$ - $\log_{10}$ plot of average length of an instar vs. average dry weight indicated that

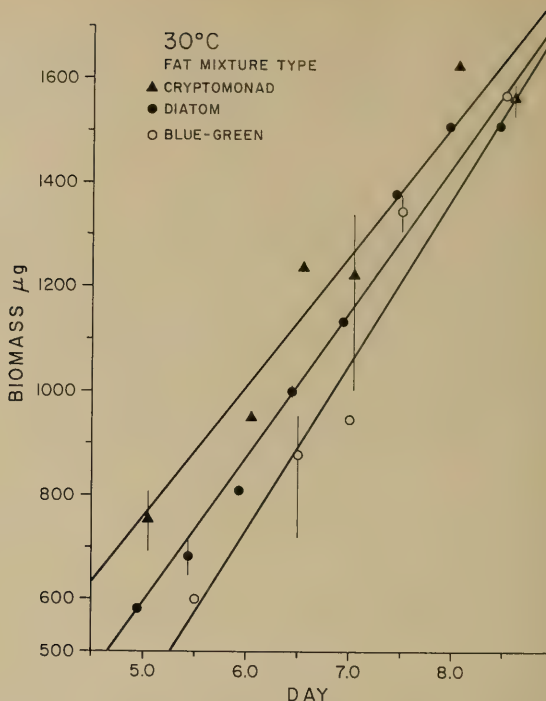


FIGURE 1. Fatty acid composition dependent qualitative effect. 30° C; diets containing cryptomonad, diatom, and blue green fatty acids at 3 mg%.

the dry weight of *Moina* increases as the cube of the body length. The equation is: dry weight = $1.1 \text{ body length}^3$; corr. = 0.9992. The total biomass of a population of *Moina* was then determined by converting lengths of each individual comprising the population into dry weight and summing the total.

Productivity was determined by dividing the total biomass of an harvested population by the total number of days since the appearance of the first brood of the original female inoculum. The day when the inoculum's first brood appeared was considered day one in order to eliminate some of the observed individual variability associated with time to maturity and thereby provide comparable data among samples of the same medium.

Many of the populations analyzed contained males and ephippial females but their presence did not affect productivity calculations since they invariably had not attained sexual maturity when the populations were harvested.

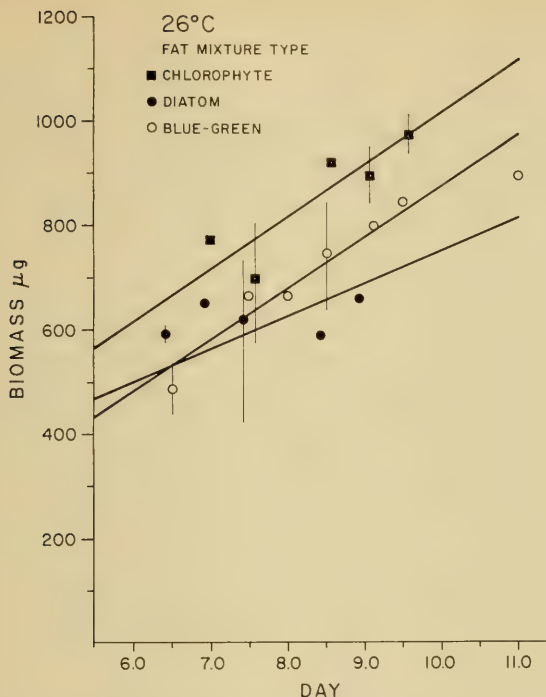


FIGURE 2. Fatty acid composition dependent quantitative effect. 26° C; diets containing chlorophyte and blue green fatty acids at 3 mg% and diatom fatty acids at 1.5 mg%.

All experimental populations were incubated in darkness. This procedure was used to eliminate the photoperiod factor which has been associated with the transition to sexuality in some species of Cladocera and to prevent the photodegradation of riboflavin, a required vitamin present in all media. Exposure to light was minimal and occurred only when cultures were shaken or when optical transmission measurements were taken. The source of light was indirect, consisting of a 10 watt light bulb fitted into a photographic safelight stand which was covered with red acetate film. Within the optical transmission apparatus red acetate material covered the slit (passage) between the light source and the area where the culture tube was positioned for a measurement. Light transmitted by the red acetate material was 600 to 700 nanometers. Past research indicates that Cladocera are minimally responsive to wavelengths in this region in both visual and nonvisual sensitivity (Scheffer, Robert, and Medioni, 1958).

RESULTS

Particles

Two types of particles were used as food in all media. It was important, therefore, to determine their size distribution. Although formulated separately, starch-protein and lipid-protein particles did not differ in size distribution. Particle size ranged from 2 to 20 μ^3 with the majority of particles (80%) in the 2 to 10 μ^3 range. Because of the similar size distributions it was assumed that there was no different sedimentation rate which could affect the probabilities of ingestion of the two types of particles.

Effects of dietary fatty acids and temperature on productivity

A series of experiments was done to define the effect of varying the quantity and quality of dietary fatty acids at different temperatures. Results of the experiments are shown in Figures 1, 2, and 3. Points of graphs represent either a single determination or an arithmetic average of multiple (2-4) determinations of biomass of populations developed on particular diets at selected days of harvest. Points derived from multiple observations include the range of values (vertical bars). Computed regression lines are included. Mortality within populations did not affect productivity determinations since, in most cases, the maximum length of an experimental period never exceeds the life span of *Moina*. If mortality was observed the population was discarded.

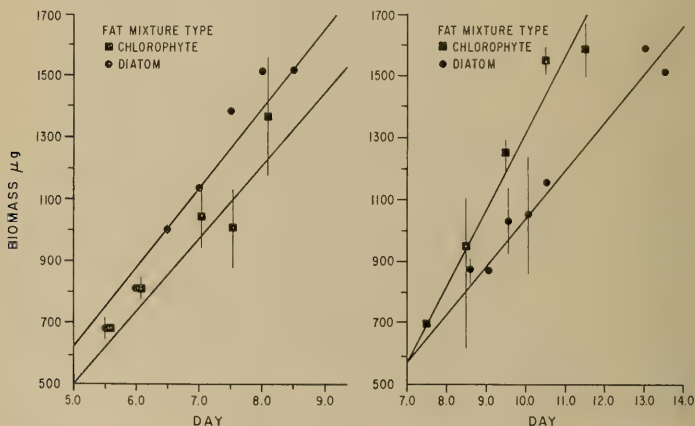


FIGURE 3. Fatty acid composition dependent temperature effect. Diets containing chlorophyte and diatom fatty acids at 3 mg%, 30° C and 22° C.

TABLE III

Productivity ($\mu\text{g/day}$, $\bar{x} \pm \text{s.d.}$)—temperature relationships; diets containing green or cryptomonad fatty acids at 3 mg%. Initial particle concentration = $540 \times 10^3/\text{ml}$, (N) = number of observations.

Diet	Fatty acid concentration	Temperature			
		30° C	26° C	22° C	14° C
Cryptomonad Blue green	3 mg%	173.4 $\pm$ 22.4 (11)	163.4 $\pm$ 31.1 (13)	122.4 $\pm$ 32.5 (10)	58.0 $\pm$ 7.1 (12)
	3 mg%	155.0 $\pm$ 31.6 (11)	151.3 $\pm$ 27.3 (10)	108.4 $\pm$ 19.6 (12)	51.4 $\pm$ 5.5 (11)

TABLE IV

The relationship of productivity ($\mu\text{g/day}$, $\bar{x} \pm \text{s.d.}$) and concentration of dietary fatty acids. Diets containing chlorophyte fatty acids, 3 and 6 mg% at 22° C. Diets containing cryptomonad fatty acids at 1.5 mg%, 3.0 mg% and 6 mg% at 26°, 22°, and 14° C. Initial particle concentration = $540 \times 10^3/\text{ml}$, (N) = number of observations.

Diet	Fatty acid concentration	Temperature		
		26° C	22° C	14° C
Cryptomonad Cryptomonad Cryptomonad Chlorophyte Chlorophyte	1.5 mg%	89.2 $\pm$ 12.7 (13)	89.4 $\pm$ 15.7 (11)	36.0 $\pm$ 7.7 (13)
	3.0 mg%	163.4 $\pm$ 31.2 (13)	122.4 $\pm$ 32.5 (10)	49.2 $\pm$ 8.0 (12)
	6.0 mg%	127.0 $\pm$ 14.7 (11)	93.1 $\pm$ 10.3 (10)	35.8 $\pm$ 6.4 (11)
	3.0 mg%		125.1 $\pm$ 20.1 (11)	
	6.0 mg%		113.4 $\pm$ 14.0 (13)	

Figure 1 illustrates the qualitative effect of dietary fatty acid composition. At 30° C and 3 mg% total fatty acids, a diet containing cryptomonad-type fatty acids is more productive than those of diatom and blue green type fatty acids. Figure 2 depicts the quantitative effect of dietary fatty acid composition. In this experiment the three diets were supplied at one-half the normal total particle concentration and the content of total fatty acids per particle was varied. At 26° C the diatom type fatty acid diet containing 1.5 mg% of fatty acids is as productive as the blue green type diet which contains 3.0 mg% fatty acid. The chlorophyte fatty acid diet at 3.0 mg% fatty acid concentration is more productive than both. Hence at 26° C the qualitatively poor blue green diet barely equalled the productivity of the qualitatively better diatom diet even with twice the total calorific content.

Figure 3 exemplifies the effect of temperature in relation to the quality of dietary fatty acids. At 30° C and 3 mg% fatty acid concentration, the diet containing diatom fatty acids is slightly more productive than the diet containing the chlorophyte fatty acids. This relationship is inverted at 22° C.

One way ANOVA indicates that productivity differences between chlorophyte (3 mg%) and diatom (1.5 mg%) fatty acid diets at 26° C, the chlorophyte (3 mg%) and blue green (3 mg%) fatty acid diets at 26° C and the chlorophyte (3 mg%) and diatom (3 mg%) fatty acid diets at 22° C are significant ($P < 0.05$).

The productivity of every diet decreased as the incubation temperature decreased. This relationship is illustrated in Table II where productivity values of a cryptomonad fatty acid diet at 3 mg% fatty acid and a blue green fatty acid diet at 3 mg% fatty acids are listed at five different temperatures, 30°, 26°, 22°, 18°, and 14° C. One way ANOVA indicates that differences between these diets at 22° and 18° C are significant ($P < 0.05$).

Increasing the total amount of fatty acids per particle in diets while keeping the starch-protein ratio constant at 1.5:1 had an effect on productivity at the three different temperatures studied. Three diets were made to contain cryptomonad-type fatty acids in concentrations of 1.5, 3.0 and 6.0 mg% respectively. Table III shows that at those temperatures investigated, 22°, 18°, and 14° C, productivity increased from 1.5 to 3.0 mg%. However, an increase to 6.0 mg% reduced productivity, indicating that a too high dietary fat content is inhibitory. An increase in total fatty acids from 3.0 to 6.0 mg% in the chlorophyte type fatty acid diet was also inhibitory at 22° C.

DISCUSSION

Axenic culture of *Moina macrocopa* in almost chemically defined biphasic media has allowed a precise analysis of some dietary factors affecting its population dynamics, and particularly a better understanding of the combined effects of abiotic and biotic factors. It has been established that the chemical composition of food, particularly the type and content of fatty acids, can directly affect productivity. Qualitative fatty acid differences in the diets were formulated in an attempt to simulate the general composition that is unique to each of four orders of algae, Cyanophyceae, Chlorophyceae, Bacillariophyceae, and Cryptophyceae. Variations

in productivity amongst the diets can most probably be attributed to the combined effects of differences in generation time, brood size, and time between broods. The comparative quality of a particular diet can also be temperature dependent.

The poor quality, in general, of those diets containing Cyanophyceae-type fatty acids may partially explain the observed poor nutritional value of some species of blue-green algae (Arnold, 1971). A partial explanation of the different nutritiousness of various species of algae for zooplankton species may in fact be the qualitative and quantitative content of dietary fatty acid. Though no fatty acid analyses were performed on *Moina* organisms grown on particular diets, the notable differences in productivity of the various diets suggest that body fatty acids are derived entirely from dietary sources. The qualitative fatty acid composition of the lipids of *Moina* has been found to be significantly affected by diet (Watanabe, Arakawa, Kitajima, Fukusho, and Fujita, 1978). It appears that the zooplankton's capacity for efficient lipid biosynthesis or inter-conversion is poor. Fatty acids were found to be essential to fertility in *Moina* (Conklin and Provasoli, 1977).

The results of the research conducted with increased fatty acid quantities (1.5 mg%-3 mg%-6 mg%) suggest that subtle interrelationships can exist amongst macronutrient dietary components. Little or no increase in the productivity of *Moina* was attained by increasing the total cryptomonad fatty acids concentration from 3 to 6 mg% at the different temperatures. The same results occurred for a chlorophyte type fatty acid diet at 22° C. Protein and carbohydrate concentrations remained constant in these experiments. Such observations add to the convincing evidence that crustaceans cannot tolerate high levels of dietary lipid. Andrews, Sick, and Baptist (1972) demonstrated that a dietary lipid supplement (1/3 beef tallow, 1/3 corn oil, and 1/3 menhaden oil) at levels >10% adversely affected growth and survival in the shrimp *Penaeus setiferus*. Forster and Beard (1973) supplemented a shrimp meal based diet for *Palaeomon serratus* with 7.5 and 15% levels of cod liver and corn oil. At the 15% level significant growth inhibition was observed for both lipid sources. The inhibitory effects of increased fatty acids in the diet may also partially explain the nutritional inadequacy of senescent algal cells. Twenty-five percent of the total dry weight of these cells is known to be fat (Fogg, 1965).

Optimal productivity of *Moina macrocopa* requires the presence of macronutrients in the proper proportions. Provasoli and D'Agostino (1969) showed that optimal starch-protein ratios for the growth of *Artemia salina* were 5:1 and 10:1. A 1:1 ratio was inhibitory. Hence, the potential quality of food sources cannot be entirely evaluated from considerations of calorific content.

The interesting physiological responses of *Moina* to varied nutrition permit some speculation. It seems plausible to assume that given the same food, the growth and reproductive capacities of species of zooplankton can be entirely different. Zooplankton community structure in an aquatic system may in part be determined by competitive processes whose outcome is based upon the satisfaction of nutritional requirements and/or the most efficient systems of biochemical assimilation and conversion.

The subtle nutritional interrelationships between predator and prey must be

identified if successful continuous culture of other phagotrophic invertebrates like *Moina* is to be realized. With a proper understanding of nutritional requirements and efficient energy budgets, the potential husbandry of marine particulate feeders such as lobsters, oysters, scallops, and shrimp can be greatly enhanced. Knowledge of crustacean nutrition may permit the use of cladoceran cultures as an alternative secondary sewage treatment. Filter feeding of particulates by the Cladocera would eliminate much of the potential biological oxygen demand (BOD) of primary treatment effluent and resulting populations could be harvested and used as fish food.

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SUMMARY

1. The cladoceran, *Moina macrocopa americana* was cultured axenically on an artificial diet consisting of a particulate and soluble phase. The effect of changes in the quantitative and qualitative dietary fatty acid composition was investigated.

2. Qualitative fatty acid differences were made to simulate the unique proportional differences found among four orders of algae, Cyanophyceae, Chlorophyceae, Cryptophyceae, Bacillariophyceae.

3. The quality of dietary fatty acids available to *Moina* exerts an effect upon productivity and the nutritional value of a particular diet in relation to fatty acid composition can be temperature dependent.

4. Increased levels of fatty acids in the diet of *Moina* reduces productivity.

5. The cladoceran, *Moina macrocopa americana*, may be entirely dependent upon diet for its source of fatty acids.

6. A partial explanation for the differential nutritiousness of particular species of algae may be their qualitative and quantitative fatty acid content.

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DIFFERENCES IN STARFISH OOCYTE SUSCEPTIBILITY TO POLYSPERMY DURING THE COURSE OF MATURATION¹

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It is known that the eggs of marine invertebrates such as the sea urchin and starfish lose the capability to fertilize or to develop normally when they are maintained in sea water for too long a period after being shed (Goldforb, 1935; Clark, 1936). In starfish oocytes, the optimum period for insemination is between germinal vesicle breakdown and the formation of the first polar body (Fol, 1879; Delage, 1901); insemination after the formation of the first polar body results in a decreased rate of subsequent normal development (Lillie, 1915).

Some earlier investigators reported that starfish oocytes, when inseminated in sea water for several hours, lose their capacity to resist polyspermy (Chambers, 1923; Clark, 1936). Thus, the decreased rate of normal development after the formation of the first polar body may be related to polyspermy. On the other hand, Chambers and Chambers (1949) have suggested that during the course of oocyte maturation, there is a specific period of ripening of cytoplasm when insemination would result in normal fertilization and development; after this period there is a decline in proper functional interrelation between the sperm and cytoplasm.

The present paper reports findings in support of the work of Chambers (1923) and Clark (1936).

MATERIALS AND METHODS

Oocytes from *Asterina pectinifera* and *Asterias amurensis* were used. The starfish were collected at the seashore near the Marine Biological Station of Tohoku University, Asamushi. Specimens of *Asterina pectinifera* were collected in September and kept in an aquarium supplied with circulating cold sea water at 10 to 13° C for two months. Specimens of *Asterias amurensis* were collected in April and maintained in running sea water tanks at 7 to 9° C for a month in the laboratory.

Asterina pectinifera. All but a few of these oocytes showed no conspicuous change when isolated from the ovary in sea water. The isolated full-grown oocytes with germinal vesicles were prepared by tearing the ovaries with forceps in sea water and washed three times with sea water. The oocytes were kept for at least 1 hr to make sure that they did not undergo spontaneous maturation.

Asterias amurensis. These oocytes usually undergo spontaneous maturation when

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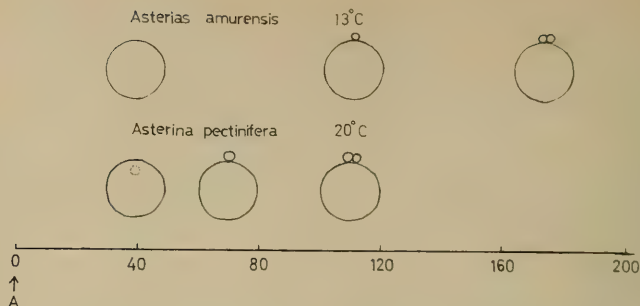


FIGURE 1. The time course of oocyte maturation in *Asterina pectinifera* and *Asterias amurensis*. This shows the time (min) when germinal vesicle breakdown occurs, and the first and second polar body form. 1-Methyladenine was added at A.

isolated in sea water. To obtain oocytes, ovaries were directly immersed in 1-methyladenine sea water. Full-grown oocytes which began germinal vesicle breakdown were spawned and these oocytes were used.

1-Methyladenine. 1-Methyladenine (Sigma) dissolved in deionized water at a concentration of 10^{-3} M was served as stock solution, and diluted with sea water to a concentration of 10^{-6} M before use.

Schedule of oocyte maturation. As shown in Figure 1, in an oocyte of *Asterina pectinifera*, the germinal vesicle disappears completely 40 min after being placed in 1-methyladenine sea water (10^{-6} M) and the first polar body forms at 65 to 75 min and the second polar body at 105 to 115 min, respectively at 20° C. In *Asterias amurensis*, oocytes which began germinal vesicle breakdown were released from ovary 40 min after ovary was immersed in 1-methyladenine sea water (10^{-6} M). The first polar body forms at 105 to 115 min and the second polar body at 170 to 180 min at 12° C.

Spermatozoa. To obtain a sperm suspension, an isolated testis was torn with forceps in an empty Petri dish. Before use, 10 μ l of semen was sucked up into a micropipette and diluted into 10 ml of 10^{-4} M histidine sea water to increase the sperms' motility. These sperm suspensions were diluted from 10-fold to 100,000-fold with fresh sea water and the number of spermatozoa was counted with haematometer. Inseminations were carried out by mixing 9 ml of oocyte suspension with 1 ml of sperm suspension.

Cytological procedures. Examined oocytes were fixed with Carnoy's solution and were sectioned by the usual paraffin method. The sections, 10 μ in thickness, were stained with Feulgen's reaction and counterstained with fast green.

Experimental procedures. The capacity of oocytes for normal development was investigated as follows; oocytes of *Asterina pectinifera* were used. Isolated immature oocytes were immersed in 1-methyladenine sea water (10^{-6} M) at 20° C and then, one part of these oocytes was transferred to another Petri dish containing fresh sea water and inseminated at one period, the other part was transferred to another Petri dish and inseminated at another period, respectively, so on. Oocytes were 2000 to 2500 per 10 ml sea water and concentration of sperm was finally 2×10^6 per ml. The number of the oocytes which formed fertilization membranes was counted 5 min after each insemination and then these oocytes were kept for 20 hr at 20° C. The oocytes were allowed to develop to early gastrula and the number was counted under a light microscope.

The occurrence of normal first cleavage was checked as follows: the ovaries of specimens of *Asterias amurensis* were directly immersed in 1-methyladenine sea water (10^{-6} M) and one part of released oocytes (2000–2500) was transferred to Petri dishes containing 10 ml fresh sea water at 40, 110, 150, and 180 min after the immersion in 1-methyladenine sea water and inseminated respectively. The concentration of sperm used was finally 8×10^5 per ml. Formation of fertilization membrane and first cleavage were observed 2.5 hr after each insemination. This experiment was performed at 12° C.

The relation between occurrence of abnormal cleavage and sperm concentration was examined as follows: oocytes of *Asterias amurensis* (2000–2500) were transferred to Petri dishes containing 10 ml fresh sea water at 40 and 180 min after addition of 1-methyladenine sea water (10^{-6} M) and inseminated with various sperm concentrations which were from 8×10^1 to 8×10^6 per ml, respectively. Formation of fertilization membrane and first cleavage were checked at 2.5 hr after each insemination. This experiment was done at 12° C.

RESULTS

Oocytes of *Asterina pectinifera* were immersed in 1-methyladenine sea water for various periods ranging from 40 to 180 min. The oocytes were inseminated in fresh sea water, at various intervals, after being placed in 1-methyladenine sea water. The formation of fertilization membrane was checked 5 min after insemination, and then the oocytes were continuously kept in sea water for 20 hr; at the end of the experiment the number of oocytes which developed to early gastrula was counted under a light microscope.

When inseminations were performed between 40 and 80 min after being placed in 1-methyladenine sea water, 73% of the oocytes developed to early gastrula. Forty-five per cent developed to gastrula, when inseminated at 120 min (period of the formation of second polar body) and only 2% at 180 min, respectively (Fig. 2).

Usually, germinal vesicle breakdown occurs spontaneously in a few per cent of the oocytes after isolation in sea water without 1-methyladenine application in *Asterina pectinifera* oocytes. In order to synchronize the course of oocyte maturation, isolated oocytes were kept in sea water for 1 hr and then spontaneously matured oocytes were checked. In this experiment, the mean rate of spontaneous maturation was 19%. For these spontaneously matured oocytes, about 100 min had

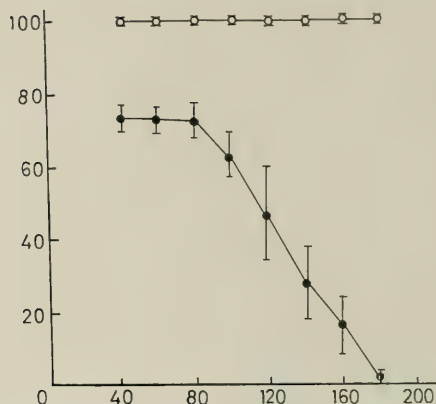


FIGURE 2. The percentage of fertilization membrane formation and normal early gastrula in *Asterina pectinifera* oocytes. Abscissa: time (min) after 1-methyladenine application; ordinate: the rate (%) of fertilization membrane and normal early gastrula formation. Open circles, fertilization membrane formation; closed circles, normal early gastrula. Each point shows mean $\pm$ of five experiments.

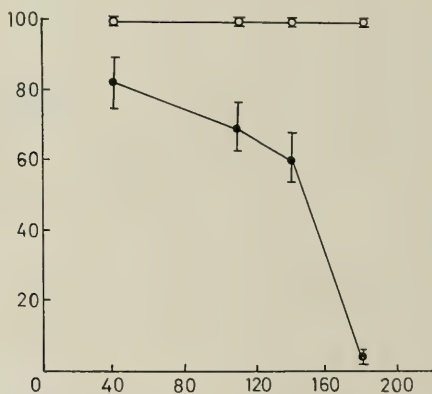


FIGURE 3. The percentage of fertilization membrane formation and normal first cleavage in *Asterias amurensis* oocytes. Abscissa: time (min) after 1-methyladenine application; ordinate: the rate (%) of fertilization membrane formation and normal first cleavage. Open circles, fertilization membrane formation; closed circles, normal first cleavage. Each point shows mean $\pm$ of four experiments.

already elapsed after isolation in sea water, before insemination, and these spontaneously matured oocytes developed abnormally upon insemination. Subsequently, the maximum rate of oocytes which developed to early gastrula did not exceed 73% even in case of the insemination at 40 min.

Next, the occurrence of first cleavage was checked 2.5 hr after insemination. In this experiment, oocytes of *Asterias amurensis* were used. After being placed in 1-methyladenine sea water for various times, oocytes were transferred to fresh sea water and inseminated. Although the formation of fertilization membrane occurred in all oocytes, the rate of normal first cleavage decreased with increased intervals after being placed in 1-methyladenine sea water: 82%, when inseminated at 40 min, 69% at 110 min (period of first polar body formation) and only 4% at 180 min (period of second polar body formation) (Figs. 3, 4).

In addition, these abnormal cleavages occurred even if 1-methyladenine was completely removed by washing with fresh sea water after occurrence of germinal

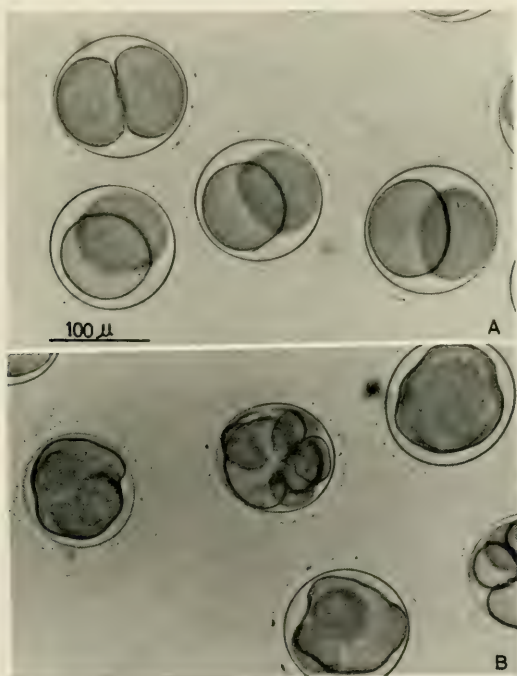


FIGURE 4. Normal first cleavage and abnormal cleavage in *Asterias amurensis* oocytes when inseminated at 40 min (A) and 180 min (B) after 1-methyladenine application.

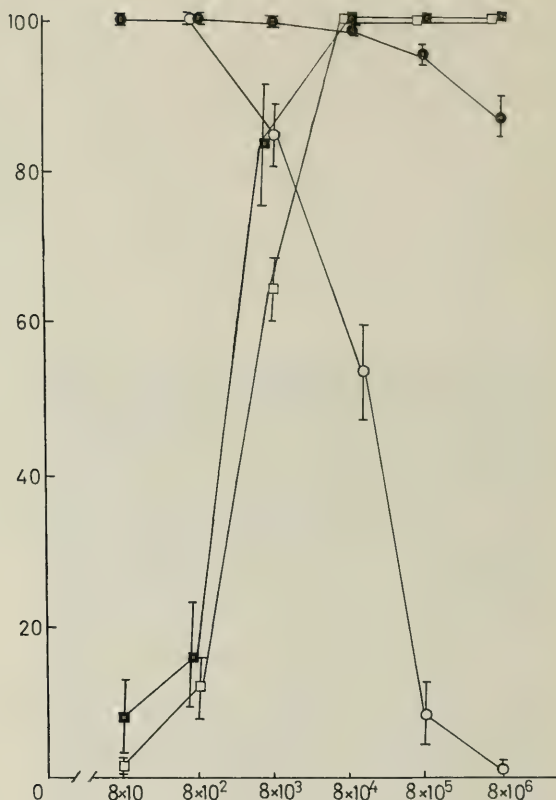


FIGURE 5. Relation between sperm concentration and normal first cleavage. Abscissa: sperm concentration (per ml); ordinate: the rate (%) of fertilization membrane formation and normal first cleavage. Solid squares, fertilization membrane formation upon insemination at 40 min; open squares, fertilization membrane formation upon insemination at 180 min; closed circles, normal first cleavage upon insemination at 40 min; open circles, normal first cleavage upon insemination at 180 min. Each point shows mean $\pm$ of five experiments.

vesicle breakdown. Therefore, this abnormality was not due to the excess of 1-methyladenine.

Next, oocytes of *Asterias amurensis* were inseminated at 40 and 180 min after being placed in 1-methyladenine sea water, with various sperm concentrations ranging from 8×10^3 to 8×10^6 per ml. When inseminations were performed

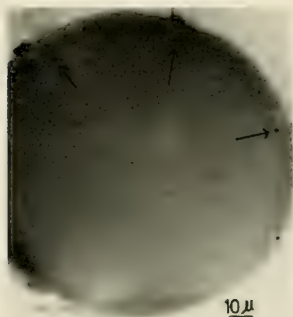


FIGURE 6. Section through an *Asterina pectinifera* oocyte which was inseminated at 180 min after 1-methyladenine application. Arrows show sperm nuclei.

40 min after being placed in 1-methyladenine sea water, the rate of fertilization membrane formation increased with the increased sperm concentrations. The rate of normal first cleavage showed only a slight decrease with higher sperm concentrations (Fig. 5); 87% of these oocytes cleaved normally when inseminated with heavy sperm concentration (8×10^6 per ml). On the other hand, when inseminations were performed at 180 min (period of second polar body formation), the rate of normal first cleavage showed a sharp decrease accompanying the increased sperm concentrations, although the rate of fertilization membrane formation showed a similar curve seen at 40 min; the rate of normal first cleavage was 53%, with moderate sperm concentration (8×10^4 per ml) which brought normal cleavage for almost all oocytes that were inseminated at 40 min, 8% with 8×10^5 per ml and only 1% with heavy sperm concentration (8×10^6 per ml). These results showed that the increase of abnormality in the oocytes inseminated at 180 min was due to the increased sperm concentration. Therefore, it seemed that polyspermy occurred in the oocytes inseminated at 180 min. This probability was confirmed cytologically (Fig. 6). Oocytes of *Asterina pectinifera* were inseminated 120 min after being placed in 1-methyladenine sea water with a moderate sperm concentration (5×10^5 per ml), and 5 min after insemination they were fixed with Carnoy's solution. Microscopic examination showed that most oocytes contained more than two sperm nuclei.

In addition, when insemination was performed with moderate sperm concentration (8×10^4 per ml) at the period of second polar body formation, the rate of fertilization membrane formation did not attain one hundred per cent (Fig. 5), but about 50% of the fertilized oocytes showed normal first cleavage and most of them developed to early normal bipinnaria.

DISCUSSION

During the course of starfish oocyte maturation, the optimum period for insemination is between germinal vesicle breakdown and first polar body formation

(Fol, 1879; Delage, 1901). Insemination after first polar body formation results in a tendency toward a decline of normal development; abnormality becomes more pronounced when oocytes are inseminated 30 to 60 min after second polar body formation (Lillie, 1915). But in these studies, the cause for occurrence of abnormality was not clarified. In our results, when inseminations were performed between germinal vesicle breakdown and first polar body formation, fertilization membrane formed normally in all cases and the first cleavage occurred normally in most oocytes. However, when inseminations were performed after first polar body formation, the rate of first cleavages and subsequent development apparently decreased. Only a small percentage of oocytes underwent normal cleavage when inseminated after second polar body formation, although fertilization membrane was formed normally in all oocytes. Figure 5 shows the increased rate of abnormal cleavage accompanying increased sperm concentrations in oocytes with second polar body: with moderate sperm concentration (8×10^4 per ml) the rate of normal cleavage was 53%, but with high sperm concentration (8×10^6 per ml), the rate of normal cleavage was only one per cent. However, even at the same high sperm concentration, normal cleavage occurred in 87% of the oocytes which completed germinal vesicle breakdown. Thus, these results suggested that the increased rate of abnormal cleavage was due to the occurrence of polyspermy. The occurrence of polyspermy was confirmed by cytological observation.

Chambers and Chambers (1949) have suggested that there is a decline in proper functional interactions between sperm and egg cytoplasm when insemination is performed after the optimum ripening period. In the present study, however, some fertilized oocytes, which were inseminated after second polar body formation, showed normal cleavage and developed to early bipinnaria if the sperm concentration was light. It is suggested that as long as polyspermy was prevented, the oocyte remained viable, even after second polar body formation.

Earlier investigators reported that starfish oocytes become susceptible to polyspermic fertilization after standing in sea water less than two hrs (Chambers, 1923; Clark, 1936). In their studies, the occurrence of polyspermy and abnormal development were not considered in relation to various periods of oocyte maturation. Our study confirms that the number of starfish oocytes showing polyspermy gradually increases with increasing time intervals between first polar body formation and sperm addition, and that almost all the oocytes show polyspermy when the insemination is performed after second polar body formation. Even in the latter case, however, fertilization membrane is normally formed. It is postulated that polyspermy occurs before fertilization membrane formation and that there is some mechanism(s) of protection against polyspermy between the first sperm entry and fertilization membrane formation. The mechanism(s) may be gradually lost after first polar body formation and disappear completely after second polar body formation.

On the other hand, polyspermy occurs regardless of the fertilization membrane formation in immature oocytes which have intact germinal vesicles (Cayer, Kishimoto and Kanatani, 1975; Hirai, 1976). Thus, the protective mechanism(s) against polyspermy may be acquired during the maturation process.

In sea urchin eggs, it was recently demonstrated by Jaffe (1976) that polyspermy was quickly inhibited as a result of electrical depolarization brought about

by the entry of the first spermatozoon. In starfish oocytes, it was reported that potassium conductance of oocyte membrane changed during maturation, thus altering the form of action potential subsequently generated (Miyazaki, Ohmori and Sasaki, 1975). Potassium conductance was small before first polar body formation, and larger after it (Miyazaki, Ohmori and Sasaki, 1975). It is suggested that the change of potassium conductance during oocyte maturation may affect electrical depolarization brought about by the entry of the first spermatozoon. Further examination would be desirable.

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SUMMARY

During the course of starfish oocyte maturation, the optimum period for insemination is between germinal vesicle breakdown and the first polar body formation. The rate of polyspermy increases with increasing time intervals between the first polar body formation and sperm addition, although the fertilization membrane is formed normally in all oocytes. As long as polyspermy is prevented, however, the oocytes remain viable even after the formation of second polar bodies. Thus, it is postulated that there is some mechanism(s) of protection against polyspermy between the first sperm entry and the fertilization membrane formation. The mechanism(s) may be gradually lost after the first polar body formation and disappear completely after the second polar body formation.

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REPRODUCTION AND DEVELOPMENT OF THE HERMAPHRODITIC SEA-STAR, *ASTERINA MINOR* HAYASHI

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A number of papers have been published on the development of sea-stars belonging to the genus *Asterina*, but the entire process of the development from eggs to juveniles is known only in four species: *A. gibbosa* (Ludwig, 1882; Goto, 1898; MacBride, 1896), *A. burtoni* (James, 1972), *A. coronata japonica* (Komatsu, 1975) and *A. batheri* (Kano and Komatsu, 1978). Among these, description of *A. burtoni* is quite brief and information on the development of other asterinids is fragmental.

Sea-stars generally are gonochoric, but a few hermaphroditic asterinid species, namely *A. batheri*, *A. gibbosa*, *A. scobinata* and *A. pancerii* (Ohshima, 1929; Delavault, 1966; Dartnall, 1970) have been known. The details of breeding in these hermaphroditic species, however, are not well documented, although the gonadal structure and development of some species like *A. gibbosa* (MacBride, 1896) and *A. batheri* (Ohshima, 1929; Kano and Komatsu, 1978) have been reported in detail.

The present study was initiated to determine the breeding and development of *A. minor*, which was recently described as a new species (Hayashi, 1974). The preliminary observations revealed that the present species had a distinct breeding behavior. Moreover, it is found that *A. minor* is hermaphroditic and is able to self-fertilize. This feature being unique among sea-stars, it was felt worthwhile to have a thorough understanding of the breeding and development of this species.

The present paper describes the structure of the gonad, breeding behavior, and development throughout metamorphosis in *A. minor*. It also incorporates the observations on isolated cultures studied during the breeding season.

MATERIALS AND METHODS

Specimens of *A. minor* were collected from the undersurface of stones at the intertidal zone of Kushimoto, Wakayama Pref., Japan, on several occasions during May of 1972 to June of 1977. They were brought to the laboratory of Toyama University and kept either in groups or individually.

The development of the species from fertilization to the completion of metamorphosis was observed with the help of a dissecting microscope and an inverted microscope. Measurements of living embryos were executed with an ocular micrometer. Examination of the skeletal system was performed on alcohol-fixed specimens treated with potassium hydroxide solution.

For microscopic observation of the reproductive organs, fresh specimens were measured and weighed, and then fixed in Bouin's solution. The fixed material was

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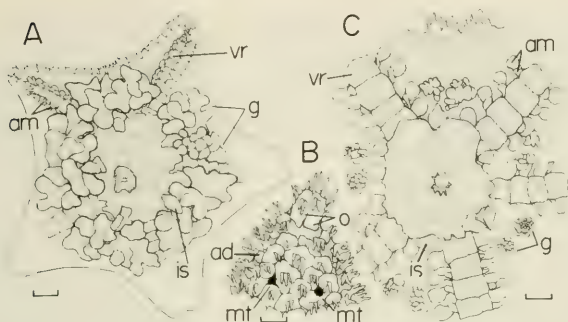


FIGURE 1. A) Aboral view of the arrangement of mature gonads in the specimen, collected on April 25, 1975, just before spawning. Aboral body wall and viscera were removed. am, ampulla; g, gonad; is, interradial septum; vr, vertebral ridge; Scale = 1 mm. B) Remnants of germinal substance, mostly egg fragments, attached to the gonopores in the specimen, collected on June 13, 1974, which was after spawning. ad, adambulacral plate; mt, remnants of germinal substance; o, oral plate. Scale = 500 μ m. C) Aboral view of the gonads in the specimen collected on June 13, 1974, which was after spawning. am, ampulla; g, gonad; is, interradial septum; vr, vertebral ridge. Scale = 500 μ m.

serially sectioned at 7 μ m by routine paraffin method and stained with Delafield's hematoxylin and eosin.

OBSERVATIONS AND RESULTS

Breeding season

In the present study, no observations were made to confirm spawning in the field. However, it is felt that a possible breeding season of *A. minor* is during May at Kushimoto. This prediction is based on the following circumstantial evidence. In the specimens examined, the gonads were largest in size and nearly mature in late April (Figs. 1A, 2A–F). In adults collected in June, some particles, possibly fragments of ova, were often observed near the gonopores (Fig. 1B). Similar particles were usually observed in the specimens spawned under laboratory conditions. The gonads of the individuals collected in June showed an atrophy and contained degenerating ova (Figs. 1C, 3A, 3B). Spawning in the laboratory as observed for 3 years occurs in the month of May (Table I). The juveniles of this species, each bearing two pairs of tube-feet in each arm, were collected in the month of June. They were estimated to be 20 to 30 days old since their fertilization. In the later months (July onward), the juveniles found in the field were larger than those collected in June (Table II).

Structure of the gonad

A pair of gonads lies at each interradial portion and each gonad is composed of cluster of tubules (Figs. 1A, 1C). Histological observations on the specimens

TABLE I

Occurrence of spawning of specimens of Asterina minor in the laboratory.

Number of animals kept together	Date of collection in the field	Time and date of the commencement of spawning in the laboratory
40	April 25, 26, 1974	21:20 May 8, 1974
2	April 25, 1975	17:05 May 2, 1975
10	April 25, 1975	13:00 May 11, 1975
4	April 25, 1975	9:20 May 24, 1975
2	Feb. 16, 1976	20:25 May 12, 1976
2	April 28, 1976	17:30 May 19, 1976
2	April 28, 1976	20:55 May 22, 1976
2	April 28, 1976	0:00 May 25, 1976
2	April 28, 1976	20:00 May 25, 1976

measuring 2.5 to 6.0 mm in R show that this species is a spatial hermaphrodite. Each gonad consists of both the ovarian tubules and the testicular ones (Figs. 2A, 2B) and has a common gonoduct which opens on the oral side of the disk (Figs. 3A, 3B). In the breeding season, the ovarian tubules show pale yellow color and the testicular tubules are whitish and semi-transparent. In general, the testicular tubules lie near the gonoduct and are smaller than the ovarian tubules located in the peripheral region (Fig. 2C). The distribution of the ovarian and testicular tubules, however, varies in different gonads and also in different individuals. In some cases, both sex elements exist in a single tubule (Figs. 2D, 2F). After spawning, all gonads become shrunken and transparent and show a green tint (Figs. 1C, 3A, 3B).

Breeding behavior

Breeding assemblage was observed in the laboratory every year. Although spawning was not observed in the field, assemblage of this species was often found

TABLE II

Number and size distribution of juveniles (under 17 pairs of tube-feet) collected in the field.

Number of tube-feet in the longest arm (in pairs)	June 10-12, 1975	July 3-6, 1974	Sept. 11-13, 1973	Feb. 16, 1976
2	10	4		
3		31	1	
4		27	5	
5		6	26	
6			32	
7			42	
8			29	
9		1	7	1
10		1	3	
11		4	5	
12		22	19	
13		36	9	
14		18	3	
15		6	2	
16		3	1	
17		2	1	

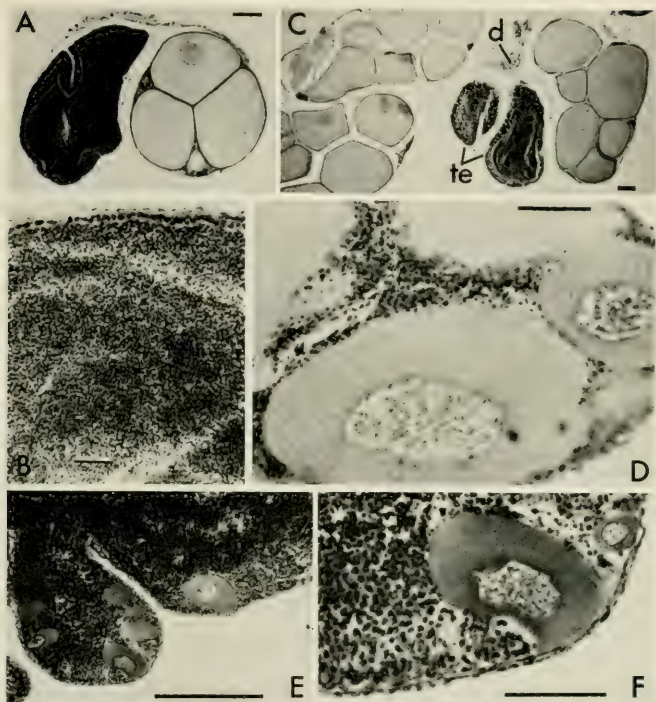


FIGURE 2. Section of the gonad of a specimen of *Asterina minor*, which was fixed just before spawning. Hematoxylin-eosin stain. A) A testicular and an ovarian tubule, containing mature sperms and ova, respectively. B) Magnified picture of the testicular tubule in Figure 2A. C) Section of gonad showing that testicular tubules (te) are situated close to the gonoduct (d). D) A hermaphroditic gonad, in which ovarian part is dominating. E) A hermaphroditic gonad, in which testicular part is dominating. F) Magnified picture of a part of Figure 2E. Scale = 100 μ m in A, C, E. Scale = 50 μ m in B, D, F.

in the field in May. The following is a description of the typical process of breeding assemblage.

Forty animals which were collected on April 25 and 26, 1974, began to organize into two groups in a large glass container in the laboratory several days after their collection. These groups were not very stable and animals moved frequently from one group to the other. Generally after about 10 days the assemblages became stable, and no animals seemed to move from their respective group. At this time animals in either group clung to one another along the margins of their bodies, or they were imbricated with others to some extent.

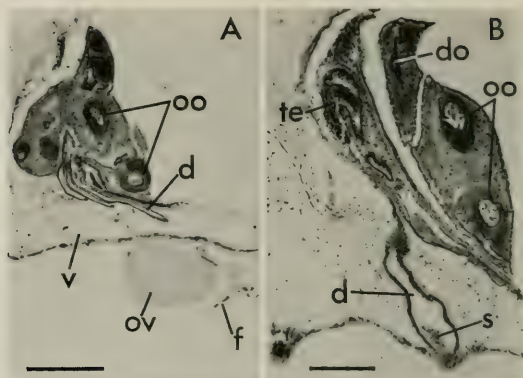


FIGURE 3. Sagittal section of the gonad of a specimen of *Asterina minor*, showing gonoduct and gonopore. A) This specimen was fixed during spawning. Note a spawned egg (ov) close to the body surface of the adult and fertilization membrane (f). d, gonoduct; oo, ovarian ova; v, ventral body wall of the adult. Scale = 50 μ m. B) This specimen was fixed just after spawning was finished. Note a remnant of sperms (s) in the gonoduct (d) and degenerating ova (do). oo, ovarian ova; te, testis. Scale = 25 μ m.

At 21:20 May 8, one animal in an assemblage comprised of 25 individuals began to spawn (a, in Fig. 4). As will be described later the eggs got attached to the substratum after their release. About 15 min thereafter, about 80 eggs were laid by this animal. At 22:50, three other animals (b, c, d, in Fig. 4) began to spawn and several minutes later each animal had delivered about 20 eggs. About 30 min thereafter, four more animals (e, f, g, h, in Fig. 4) began to spawn. Two

TABLE III

Spawning of specimens of Asterina minor in isolated culture.

Size of animal (mm) R r	Fresh weight of newly collected animal (mg)	Fresh weight of animal after spawning (mg)	Decrease of weight by spawning (%)	Number of eggs spawned	Term in isolated culture (day)
7.0 4.2	188.3	106.0	44	297	27
6.5 4.5	149.4	100.4	33	233	87
5.6 3.3	148.3	—	—	180	85
5.5 4.1	94.1	77.7	17	243	21
5.4 4.0	196.4	92.8	53	261	21
5.1 3.7	79.0	69.7	12	127	19
5.1 3.3	62.3	45.2	27	132	29
5.0 3.8	89.3	69.8	22	137	39
5.0 3.2	67.6	56.3	17	116	28
4.7 3.5	37.8	29.5	22	87	21
4.2 3.8	45.8	34.9	24	44	100
4.1 2.9	16.4	10.5	36	6	33

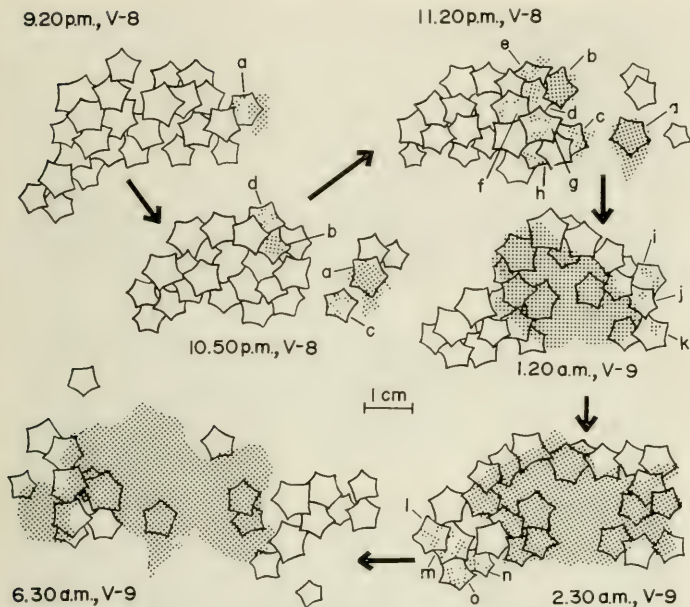


FIGURE 4. Sequential change of the distribution of individuals in a breeding assemblage. Dotted areas show the deposition of eggs. See text for detail.

hours later, three more animals (i, j, k, in Fig. 4) were found to be spawning, and soon four other animals (l, m, n, o, in Fig. 4) followed. After that, the majority of animals in this assemblage were found to be spawning. About 6 hr after the first spawning, all animals seemed to have completed spawning. At 6:30 the following morning (May 9), the assemblage was almost disordered. In the other assemblage, spawning commenced 3 hr after the onset of the spawning in the first one. The process of spawning in the other assemblage was very similar to that in the first. In the evening of May 9, the assemblages were completely disorganized and constituent individuals in either assemblage got mixed. After the disorganization of the assemblages, two egg masses were found, and these were not protected by the adults.

Isolated culture, a proof of self-fertilization

From the characteristic breeding assemblage observed, it seems that this species engages in cross-fertilization. However, there is a possibility that the species also indulges in self-fertilization, since histological observations show

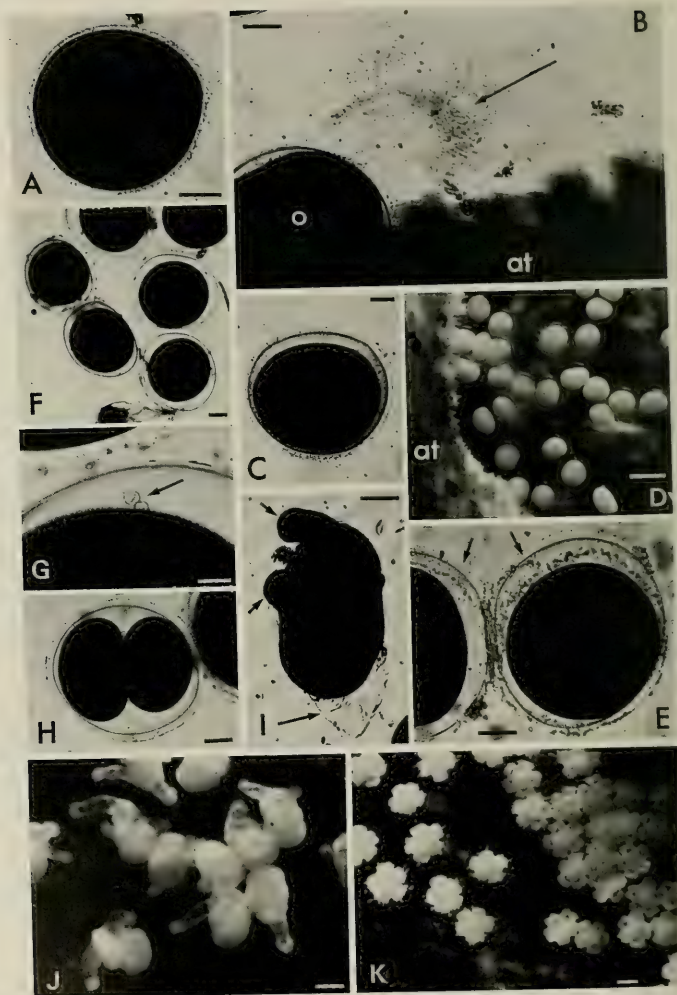


FIGURE 5. Development of specimens of *Asterina minor*. All pictures show living specimens. A) Egg just after spawning. Fertilization membrane was not yet formed. Sperms are crowding around the jelly layer. B) A mass of sperms (arrow) is seen near the spawning

both male and female elements simultaneously maturing in one individual. In order to know whether the species was able to self-fertilize, observations were made on individuals in isolated culture. Specimens, immediately after their collection in the field, were kept individually in small glass jars. Temperature during the culturing was maintained similar to that of the natural habitat, 15 to 25° C. The majority of animals in the isolated culture deposited eggs and these eggs were fertilized with the sperms ejected from the same animal in the absence of any artificial treatment. The details in respect of isolated culture and self-fertilization are given in Table III.

The eggs possibly became mature during the travel through the gonoduct or else as soon as they were released. The number of eggs spawned by one adult is related to the size of the adult. The self-fertilized eggs developed into normal embryos and became normal juveniles through metamorphosis (Fig. 5K). No differences were observed in the fertilization rates or developmental processes in the specimens held in mass culture and those kept in an isolated culture. Adults were collected in different months of the year and kept separately, but they spawned within a limited span of time in the laboratory.

The above observations demonstrate that *A. minor* has an ability to self-fertilize when kept singly. However, it is not clear whether or not self-fertilization occurs when the animal is spawning in the breeding assemblage.

Development

In this species no fertilization membrane was observed in the freshly laid eggs (Fig. 5A).

The egg is spherical with a diameter of $437 \pm 5 \mu\text{m}$ (mean $\pm$ s.e., $n = 37$). They are transparent yellow and have a jelly layer of about $20 \mu\text{m}$ thick. A few minutes after spawning, sperm masses are released from the same adult (Fig. 5B) and the sperms are dispersed. The head of the sperm is spherical, about $3 \mu\text{m}$ in diameter, and the tail is about $30 \mu\text{m}$ in length. The eggs a few minutes after spawning, attach to the substratum and the neighbouring eggs by their sticky jelly layer. Ten minutes after spawning, elevation of the fertilization membrane is recognized. Figure 5C shows fertilization membrane in an egg 30 min after spawning. The spawned eggs are laid on the bottom in one layer (Figs. 5D, 5E). About 2 hr after spawning, polar bodies are seen in the perivitelline space, which is about $50 \mu\text{m}$ in height (Figs. 5F, 5G). Three hours after spawning at 20 to 23° C, the first cleavage occurs through the animal-vegetal axis (Figs. 5H, 6A), and 40 min thereafter it is followed by the second cleavage which is perpendicular

adult (at). Fertilization membrane is being formed in an egg (o), about 10 min after spawning. C) Egg about 30 min after spawning. D) Adult (at) and eggs spawned from it, 1 hr after the commencement of spawning. E) Magnified picture of a part of Figure 5D. Two eggs are attached to each other by their jelly layers (arrows), which lie outside of the fertilization membrane. F) Eggs 2 hr after spawning. G) Magnified view of F. Note polar bodies (arrow). H) First cleavage, 3 hr after spawning. I) Hatching brachiolaria, view from the ventro-lateral (left) side. Long and short arrows indicate fertilization membrane and brachiolar arms, respectively. J) Developed brachiolariae, creeping on the substratum. K) Metamorphosed juveniles, 15 days after spawning. Scale = $50 \mu\text{m}$ in D, G. Scale = $100 \mu\text{m}$ in A, B, C, E, F, H, I. Scale = $200 \mu\text{m}$ in J. Scale = $100 \mu\text{m}$ in K.

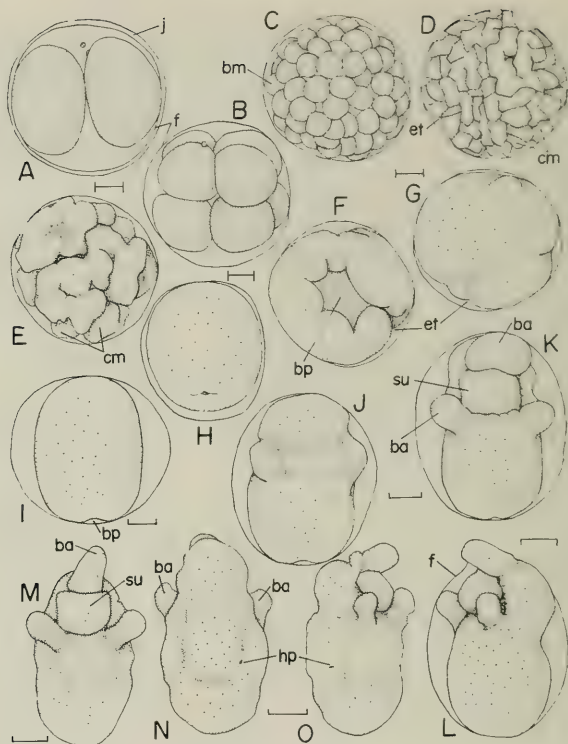


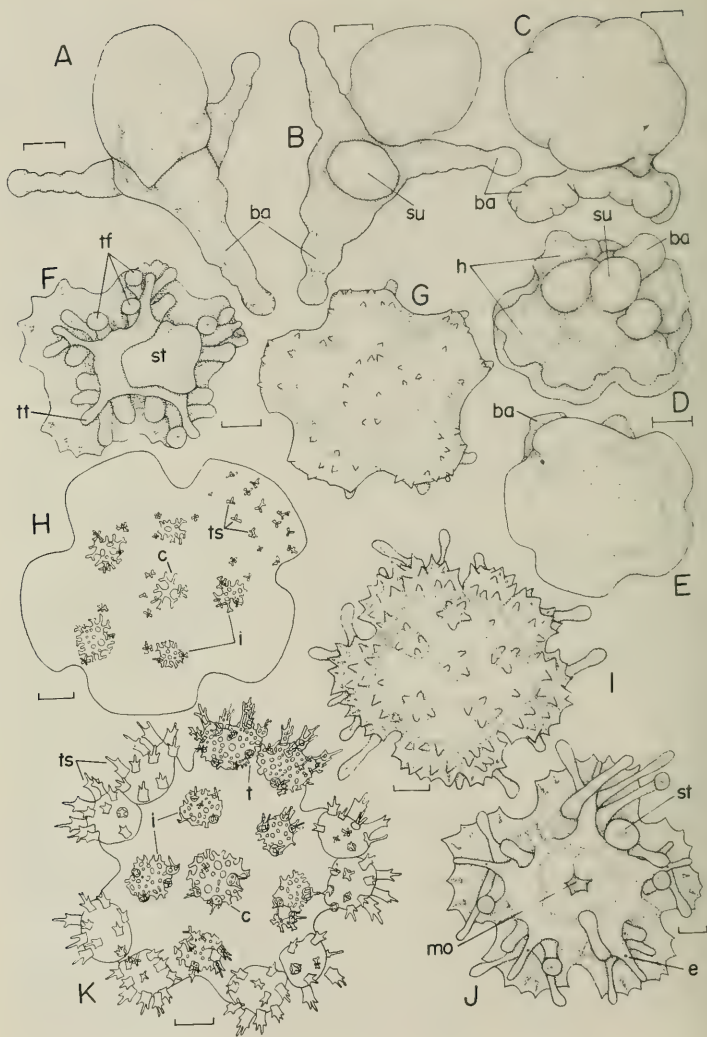
FIGURE 6. Development of specimens of *Asterina minor*. All drawings are from a living specimen, jelly layer is not shown except in A. All scales show 100 μ m. A) Two-cell stage. f, fertilization membrane; j, jelly layer. B) Eight-cell stage, 4.5 hr after spawning. f, fertilization membrane. C) 64-cell stage, 6.5 hr after spawning. bm, blastomere. D) Early wrinkled blastula, 10 hr after spawning. cm, cell mass; et, egression tract. E) The most wrinkled blastula, 12.5 hr after spawning. F) Early gastrula, 18 hr after spawning. Egression tracts (et) are still recognized on the surface of the embryo, view from the vegetal pole. bp, blastopore. G) Early gastrula, 18 hr after spawning, view from the animal pole. et, egression tract. H) Gastrula, 36 hr after spawning. I) Gastrula, 40 hr after spawning. bp, blastopore. J) Brachiolaria, 2 and a half days after spawning, view from the ventro-lateral (right) side. K) Brachiolaria, 3 days after spawning, ventral view. ba, brachiolar arm; su, sucker. L) Brachiolaria at the commencement of hatching, 4 days after spawning, view from the ventro-lateral (left) side. f, fertilization membrane. M) Brachiolaria, just after hatching, ventral view. ba, brachiolar arm; su, sucker. N) Same as M, dorsal view. ba, brachiolar arm; hp, hydropore. O) Same as M, ventro-lateral (right) view. hp, hydropore.

to the first. The cleavage is total, equal, and radial. The embryo reaches the eight-cell stage (Fig. 6B) and the 64-cell stage (Fig. 6C), 4.5 and 6.5 hr respectively after spawning. Eight hours after spawning, the embryos enter into the wrinkled blastula stage. The process of wrinkling has been earlier reported in detail (Komatsu, 1976). Figures 6D and 6E illustrate an early wrinkled blastula and most wrinkled blastula, respectively. Twenty-four hours after spawning, the wrinkled blastula stage is completed.

At the end of the wrinkled blastula stage, gastrulation by invagination takes place (Figs. 6F, 6G). Thirty-six hours after spawning, the gastrula begins to rotate within the fertilization membrane (Fig. 6H) and becomes enlarged along the archenteric axis. Figure 6I shows a gastrula 40 hr after spawning. It measures about 500 μm in length and 350 μm in width. Two days after spawning, the ventral side of the embryo becomes flattened and the rudiments of the brachiolar arms (lateral arms) appear at the ventro-lateral side of the embryo. These arms grow gradually and the third arm (anterior arm) emerges from the ventral side of the anterior portion of the embryo. Two and a half days after spawning, the embryo becomes pear shaped, with three distinct brachiolar arms and a rudimentary sucker which appears in the area surrounded by the brachiolar arms (Fig. 6J). Three days after spawning, the brachiolaria measures 550 μm in total length (Fig. 6K). The anterior half of the embryo, which bears three brachiolar arms and a sucker, corresponds to the stalk of the larva and the posterior half is the disk of the larva. Lateral arms are longer, 150 μm in length, than the anterior arm, which is about 100 μm . A blastopore and a hydropore are observed at the posterior tip and at the dorsolateral (right) side of the larva, respectively. Three and a half days after spawning, the blastopore is closed.

About four days after spawning, the brachiolaria is hatched from the fertilization membrane (Figs. 5I, 5L), usually at the anterior portion. Figures 6M, 6N and 6O show a freshly hatched larva. The present species has no pelagic life and the brachiolariae creep on the substratum with their developed brachiolar arms throughout the brachiolaria stage (Fig. 5J). One day after hatching, the brachiolariae attach to the substratum with their brachiolar arms and not with the sucker (Figs. 7A, 7B). This attachment is a sign of the commencement of metamorphosis. At this stage, the brachiolar arms are markedly long and appear semi-transparent excepting at the tip ends. The anterior arm measures 250 μm , and the lateral arms are 350 μm in length.

About 12 hr later, the disk begins to transform into a subpentagonal form (Fig. 7C), and the stalk begins to shrink rapidly. The rudiments of the tube-feet are seen on the hydrollobes, these are clearly visible in the future oral side of the adult disk. Figures 7D and 7E show a larva one day after the one shown in Fig. 7C. This larva is about 500 μm in diameter and has a hydropore in an inter-radius of the future aboral side of the disk. A degeneration of the stalk, including the brachiolar arms and sucker, progresses gradually. About 7 days after spawning, the larvae are freed from the substratum due to the extreme degeneration of the brachiolar arms. They are able to move by means of their tube-feet (Figs. 7F, 7G). On the aboral side of the larva at this stage, one central and five inter-radial plates are recognized (Fig. 7H). Metamorphosis is completed with the opening of the mouth about 10 days after spawning (Figs. 7I, 7J). At this stage,



the juvenile is 700 μm in diameter, and bears a red eye-spot at the basal portion of each terminal tentacle. On the oral side, a rudiment of the stalk is still observed. In each interradius, the formation of a pair of oral plates is noticed. Skeletal plates on the aboral side are shown in Fig. 7K. It is of interest to note that a slit is seen at the midline of each terminal plate, making it appear as if one terminal plate is composed of two pieces. Fifteen days after spawning, that is about 5 days after the completion of metamorphosis, the juveniles start moving from the place where they were laid (Figs. 5K, 8A). At this stage, each oral plate bears a spine which is pointed at the center of the mouth (Figs. 8B, 8C). On the aboral side of disk, small radial plates are being formed (Fig. 8D). After the spawning the juveniles grow to the size of 1000 and 1200 μm in diameter after 20 and 30 days, respectively. In specimens 30 days after spawning, each radial plate bears one spine at the center. In each interradius of the aboral side, one pair of the secondary plates is formed. On the oral side, a pair of adambulacral plates is formed in each arm and each adambulacral plate is furnished with one spine.

The juveniles were kept in the laboratory several months after the completion of metamorphosis, but no further development was observed beyond the stage 30 days after spawning (20 days after the completion of metamorphosis). Field surveys were then undertaken to obtain supplementary material for understanding post-metamorphic development. As shown in Table II, a number of juveniles were collected. Among these, two animals are described here in detail. One animal collected on July 3, 1974, was 1500 μm in diameter and had three pairs of the tube-feet in each arm (Figs. 9, 10, 11A). At each interradius, one pair of superomarginal plates was present, each plate having two prominent spines. On the oral side, five pairs of oral plates encircle the mouth. In each arm, two pairs of adambulacral plates and one pair of inferomarginal plates were present. The first adambulacral plate had two small spines. The second adambulacral plate was smaller than the first, and each of them had one spine. The other specimen collected on Sept. 7, 1973, was 3000 μm in diameter and possessed seven pairs of tube-feet in each arm (Figs. 11B, 11C). This specimen had well-developed aboral skeletal plates, some of which were imbricated with each other. Between the neighboring interradial plates, there was papular area, each of which had one papula. In one of the interradial plates there was a concave portion and this might be a rudiment of the madreporite. Each arm had several pairs of supero-

FIGURE 7. Development of specimens of *Asterina minor*. All drawings are made from living specimens, except H and K, which were treated with KOH solution before examination. All scales show 100 μm . A) Brachiolaria, 5 days after spawning, strongly attached to substratum with brachiolar arms (ba), dorsal view. B) Same as A, ventral view. ba, brachiolar arm; su, sucker. C) Metamorphosing larva, 12 hr after that shown in Figures 7A and B, view from the future aboral side of the juvenile. ba, brachiolar arm D) Metamorphosing larva, 1 day after that shown in Figure 7C, future oral view. ba, brachiolar arm; h, hydrolobe; su, sucker. E) Same as D, future aboral view. ba, brachiolar arm. F) Metamorphosing larva, 7 days after spawning, future oral view. st, stalk; tf, tube-foot; tt, terminal tentacle. G) Same as F, future aboral view. H) Aboral skeletal plates, in the same stage shown in Figures 7F and G. c, central plate; i, interradial plate; ts, spines on the terminal plate. I) Juvenile just after the completion of metamorphosis, 10 days after spawning, aboral view. J) Same as I, oral view. e, eye-spot; mo, mouth; st, markedly degenerated stalk. K) Aboral skeletal plates, in the same stage shown in Figures 7I and J. c, central plate; i, interradial plate; t, terminal plate; ts, spines on the terminal plate.

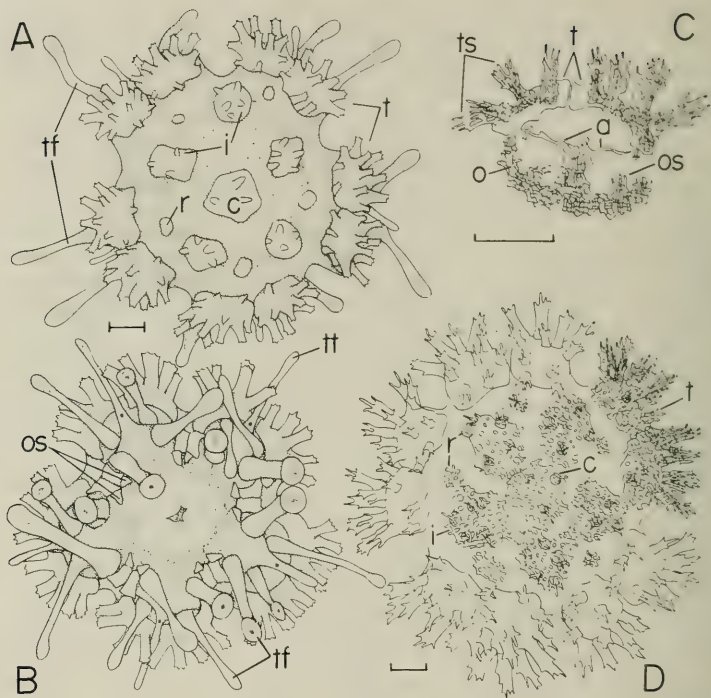


FIGURE 8. Juvenile of specimens of *Asterina minor*, five days after the completion of metamorphosis, having two pairs of tube-feet. A and B are drawn on living specimens and C and D are on KOH treated specimens. All scales show 100 μ m. A) Aboral view. c, central plate; i, interradian plate; r, radial plate; t, terminal plate; ff, tube-foot. B) Oral view. os, oral spine; ff, tube-foot; tt, terminal tentacle. C) Skeletal plates of an arm, oral view. a, ambulacral plate; o, oral plate; os, oral spine; t, terminal plate; ts, spine on the terminal plate. D) Aboral skeletal plates. c, central plate; i, interradian plate; r, radial plate; t, terminal plate.

marginal plates and inferomarginal plates, the latter both in size and number were larger than the former. Six pairs of adambulacral plates were present in each arm, and each of the three proximal plates (1st, 2nd, and 3rd) possessed three spines. In the interradian area of the oral side, there were several ventro-lateral plates in addition to adambulacral plates and inferomarginal plates. One slit was always recognizable along the midline of the terminal plate.



FIGURE 9. Aboral skeletal plates of a juvenile specimen of *Asterina minor*, with three pairs of tube-feet in each arm, KOH treated. c, central plate; i, interradial plate; r, radial plate; sm, superomarginal plate. Scale = 250 μ m.

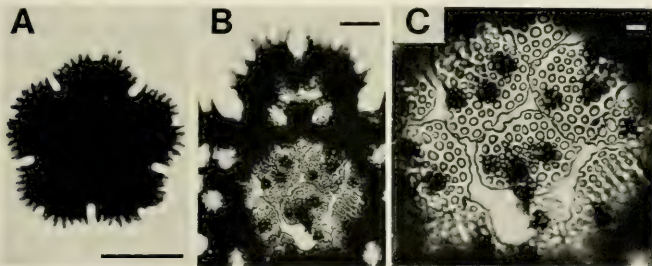


FIGURE 10. Skeletal plates of a juvenile specimen of *Asterina minor*, in the same stage shown in Figure 9, KOH treated. A) Shadow picture showing spines on terminal plates and contour of the body. Scale = 500 μ m. B) Picture of plates in one arm and disk, in the same specimen shown in Figure A. Shaded area corresponds to plates in the oral side. Scale = 150 μ m. C) Magnified picture of the central portion of Figure B. Scale = 150 μ m.

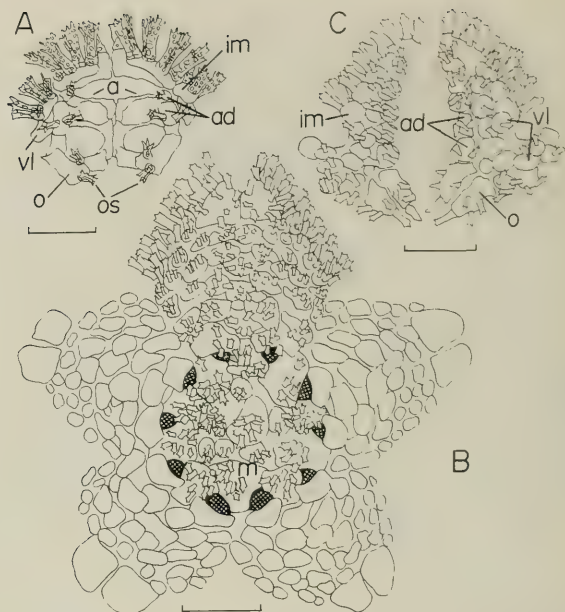


FIGURE 11. Skeletal plates of juvenile specimens of *Asterina minor*, treated with KOH. A) Oral skeletal plates in one arm in the specimen shown in Figure 9. a, ambulacral plate; ad, adambulacral plate; im, inferomarginal plate; o, oral plate; os, oral spine; vl, ventro-lateral plate. Scale = 200 μ m. B) Aboral skeletal plates of a specimen having seven pairs of tube-feet in each arm, hatched portions show papular area. m, interradial plate with a rudiment of madreporite. Scale = 450 μ m. C) Skeletal plates in the oral side of one arm in the specimen shown in Figure B. ad, adambulacral plate; im, inferomarginal plate; o, oral plate; vl, ventro-lateral plate. Scale 500 = μ m.

DISCUSSION

A. minor lacks the bipinnaria stage and develops without pelagic life as known in *A. gibbosa* and *A. exigua* (Ludwig, 1882; MacBride, 1896; Mortensen, 1921). In general, asteroids whose development is direct have pear-shaped brachiolaria with three brachiolar arms. In species belonging to *Asterina*, the brachiolar arms of *A. batheri* and *A. coronata japonica* are short and blunt (Komatsu, 1975; Kano and Komatsu, 1978). The brachiolariae of these species have pelagic life. On the other hand, the brachiolar arms of *A. minor* are well developed and resemble those of *A. gibbosa* and *A. exigua*, and yet these brachiolariae spend benthonic life creeping on the substratum before metamorphosis. It is likely, therefore, that well-developed brachiolar arms are one of the adaptive characters of the benthonic life.

As described before, asteroids, barring a few hermaphroditic species, are gonochoric (Delavault, 1966). Sexuality of asterinids has been fairly well studied since Cuénot (1887) reported the occurrence of protandric hermaphroditism in *A. gibbosa*. Among asterinid species, in addition to *A. gibbosa*, hermaphroditism is known in *A. pancerii*, *A. batheri* and *A. scobinata* (Ohshima, 1929; Cognetti, 1954; Dartnall, 1970; Kano and Komatsu, 1978). In *A. batheri* a few hermaphroditic individuals occur among gonochoric individuals (Ohshima, 1929). No difference seems to exist in the sexual status of this species from different geographical regions (Kano and Komatsu, 1978). In this study, it was found that *A. minor* is a spatial hermaphroditic and all individuals bear hermaphroditic gonads comprising both functional testes and ovaries. In the breeding season, both elements mature simultaneously and the eggs can be fertilized with the sperm released from the same individual. This study has presented for the first time evidence of definite self-fertilization in asteroids.

Chia (1968) reported that *Leptasterias hexactis* aggregates under rocks during breeding season. Such a gathering is usual behavior in brooding species (Kubo, 1951). It is interesting to note that *A. minor* shows a distinct breeding assemblage, although it is not a brooding species and is capable of self-fertilizing. These facts may imply a complex historical background through which *A. minor* has been speciated.

The data accumulated so far indicate that genus *Asterina*, or its closely related groups, despite intimate similarity in adult morphology in different species, shows a remarkable variety in the mode of development and the method of breeding (Ludwig, 1882; MacBride, 1896; Goto, 1898; Mortensen, 1921; James, 1972; Komatsu, 1975; Kano and Komatsu, 1978). This observation may bring out the importance of studies on the development of *Asterina* group which may not only clarify the ontogeny of a given species but may also help towards understanding the evolution of asteroid development as a typical group having divergent breeding methods and development.

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SUMMARY

1. The breeding season of *Asterina minor* is estimated to be during the month of May in Kushimoto, Japan. *A. minor* shows a characteristic breeding assemblage and the eggs are laid on the substratum in a mass spawning. The eggs are not protected by the adults.

2. *A. minor* is a spatial hermaphrodite, where ovaries and testes in an individual become mature simultaneously. Isolated individuals are capable of self-fertilizing and the self-fertilized eggs develop normally.

3. The spawned eggs are spherical, yellow, and 437 μ m in average diameter. They attach to the substratum with a sticky jelly layer. Cleavage is total and radial.

4. Eggs through the wrinkled blastula stage develop into a pear-shaped brachiolaria bearing three brachiolar arms within the fertilization membrane.

5. About four days after spawning, the brachiolariae hatch from the fertilization membrane and creep on the substratum with well-developed brachiolar arms. There is no evidence of pelagic life in the present species.

6. One day after hatching, brachiolariae attach firmly to the substratum with the brachiolar arm and undergo a rapid transformation of the body (metamorphic climax). Metamorphosis is completed with the opening of the mouth about 10 days after spawning. The newly metamorphosed juvenile is about 700 μ m in diameter and each arm bears two pairs of the tube-feet and one red eye-spot at the base of the terminal tentacle.

7. The reproduction and larval development of *A. minor* are unique, and the study may prove a good guideline for understanding the evolution of reproduction and development in Asteroidea.

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THE EFFECT OF SIZE, TEMPERATURE, OXYGEN LEVEL AND
NUTRITIONAL CONDITION ON OXYGEN UPTAKE IN THE
SAND DOLLAR, *MELLITA QUINQUIESPERFORATA*
(LESKE)

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Because metabolism often represents a large source of expended energy, accurate measurement of metabolism is essential for construction of energy budgets. Metabolic processes, as measured indirectly by oxygen uptake, however, may vary with physical condition and physiological state. It is often difficult to assess the results of all these variables on metabolism, and some effects are either discounted or estimated.

Such estimations may be misleading, especially in echinoids, where relatively few studies have been made and where different species of echinoids may respond differently to certain variables. The purpose of this study is to obtain a relatively accurate estimation of metabolic energy expenditure in the sand dollar, *Mellita quinquesperforata*, by measuring oxygen consumption seasonally at ambient water temperatures, under various degrees of starvation, during the day and night, and at different oxygen concentrations on various size animals.

MATERIALS AND METHODS

For monthly measurement of oxygen consumption, five or six animals of various sizes were collected from Mullet Key, a sub-tidal, semi-protected sandy beach area along the Gulf of Mexico, adjacent to the mouth of Tampa Bay, Florida (27° 38' N; 82° 44' W). After allowing the guts to clear overnight, animals were placed in individual dishes containing either 200 ml (for small animals), 500 ml (for medium-sized animals) or 1000 ml (for large animals) of freshly aerated sea water. After 0.5 hr, a 1-cm layer of mineral oil (which blocks oxygen diffusion) was poured over the surface of the water. At this time, an initial water sample was taken from a dish with no animal (control). A known volume of water (approx. 30 ml) was removed from each dish at 1 and 2 hr after sealing; the water was gently stirred just before sampling. Oxygen in water samples was measured using the micro-Winkler method (Hoar and Hickman, 1967). Sea water used for all experiments was collected from the field and filtered through 0.22 μ Millipore filters before use. A constant temperature chamber (dimly lit) stimulated monthly environmental water temperatures and fluctuated less than 0.2° C during each experiment. Salinity varied little between experiments (33.0-34.9‰). After 2 hr, each animal was removed, weighed, and placed in a drying oven at 80° C. Dried animals were weighed and ground to powder in a Wylie mill. A small sample of this material was used for measurement of total nitrogen by the micro-Kjeldahl (Holland, 1964). Monthly rates of oxygen consumption are expressed on a wet, dry and total body nitrogen weight basis. Analysis of the

data was done on values obtained after 1 hour as tests showed *M. quinquiesperforata* to be an oxygen conformer.

To ascertain how rate of oxygen consumption is affected by reduced oxygen tensions in sea water, animals of similar weight (7 g dry weight) were placed in dishes containing a known volume of sea water (100 ml) in ten experiments. The dishes were sealed with a lid through which an oxygen electrode was mounted. The rate of respiration in these closed dishes was measured every 10 minutes. The water was stirred during each reading by a magnetic stirbar located just beneath the oxygen electrode. The water temperature in all these experiments was 25° C.

In addition to monthly measurements using a closed system, the rate of oxygen consumption was measured in June in an "open" or "flow-through" system, similar to the one described in Hoar and Hickman (1967). Six animals were placed in individual dishes which were closed to general water circulation except for entrance and exit holes. Water entered the dishes and was continuously siphoned into stoppered Erlenmeyer flasks. The rate of flow through the dishes was measured each day and adjusted so that oxygen tension never fell below 80% saturation. Animals used in this experiment were acclimatized to summer water temperatures (29–33° C). Experiments were run at 15, 20, 25, 30, and 35° C. For each experimental temperature, the water in the flow-through apparatus was changed and different animals were used. Animals were not placed into the dishes until all feces had been expelled (usually overnight); oxygen readings were begun after animals had been held at experimental temperatures in the dishes for one day. Oxygen concentration was measured in the Erlenmeyer flasks using a Beckmann Field-Lab oxygen meter fitted with a platinum electrode. Readings were taken at 4-hr intervals for 48 continuous hours. The experimental temperature was maintained by placing the whole flow-through apparatus in a constant temperature chamber. A dim light switched on at 0700 hr and off at 2200 hr simulating day-night conditions. After each experiment, animals were removed from the dishes, weighed and dried as described above. Results from the flow-through system were compared with results from a closed respiratory system. One-hour closed system measurements were run at the same temperature at the beginning of each flow-through experiment.

The effect of starvation upon the rate of oxygen consumption was measured at different times of the year by starving animals in filtered sea water for periods up to 1 month. Water in the holding tanks was renewed every 2 days with freshly collected and filtered sea water. During starvation, water temperature in tanks simulated ambient temperatures. The oxygen consumption of animals which were starved for various times (5–30 days), was measured in a closed system.

Results of all oxygen uptake experiments are expressed as log-log linear regression equations with $\log_{10}$ rate of oxygen uptake and $\log_{10}$ body weight (wet, dry or body nitrogen weight) being the two variables. Slopes and intercepts from different seasons or experiments were compared using analysis of covariance (Snedecor and Cochran, 1971). Variances were checked with Bartlett's Chi-square (Snedecor and Cochran, 1971). For ease in comparing rates of oxygen consumption at different temperatures, pooled slopes were used when statistically valid and intercepts were adjusted as follows; new intercept equals average of Y values minus pooled slope times average of X values.

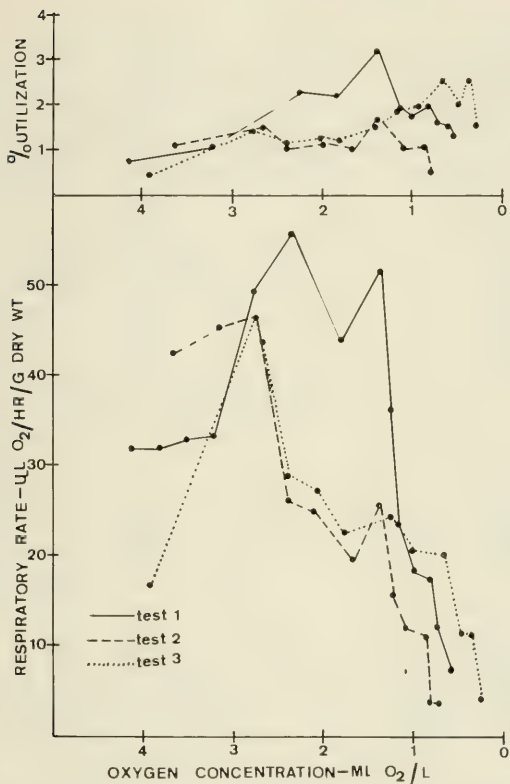


FIGURE 1. Rate of oxygen uptake ($\mu\text{l O}_2/\text{hr/g}$ dry weight) and percent utilization of available oxygen by *Mellita quinquiesperforata* in decreasing oxygen concentrations in three representative experiments. A closed system was used for measurement.

RESULTS

Rates of respiration of *Mellita quinquiesperforata* conform to oxygen concentrations in water (Fig. 1). Oxygen consumption decreases with decreasing oxygen pressure in a nearly linear manner. At very low levels of external oxygen (< 1 ml O₂/liter), a low level of respiration is maintained.

In monthly oxygen measurements at ambient temperatures, rates of respiration are dependent on both body weight (wet, dry or body nitrogen) and water

TABLE I

Summary of linear regression equations for ml O₂ respired/hr per animal vs. dry body weight in non-starved *Mollita quinquesperforata* at monthly ambient water temperatures and in starved sand dollars for various periods of starvation at ambient water temperatures. The form of the linear regression equations is $\log_{10} \text{ ml O}_2 \text{ respired/hr per animal (Y) equals } \log_{10} \text{ g dry body weight (X) times slope plus intercept}$. A closed system was used for measurement.

Date	Dry weight of animals (g)	Ambient water temperature (°C)	# days starved	N	Slope	"P" for slope	Intercept	Intercept (adjusted)
Non-starved animals								
VII/4/72	1.5-26.5	31	—	5	0.5448	180.81**	-0.7508	-0.7541
VII/25/72	0.3-17.6	30	—	6	0.5754	61.46**	-0.9960	-0.9707
VIII/30/72	2.2-16.0	33	—	6	0.5799	41.44**	-0.8954	-0.8655
IX/30/72	1.0-21.9	27	—	5	0.5174	49.88**	-0.9671	-0.9908
X/24/72	0.4-26.8	25	—	5	0.6221	308.72**	-1.0426	-1.0027
XI/15/72	1.0-15.4	22	—	5	0.5136	1167.25**	-0.9850	-1.0119
XII/15/72	2.4-15.9	20	—	6	0.5285	18.55**	-1.0551	-1.0717
I/16/73	2.0-13.8	20	—	6	0.5116	30.17**	-0.9577	-0.9876
II/27/73	4.1-12.0	15	—	6	0.6093	10.59*	-1.1714	-1.1174
V/14/73	1.7-35.8	28	—	6	0.6704	21.60**	-1.0208	-0.8706
Starved animals								
VI/30/72	0.6-54.6	30	5	5	0.8410	56.02**	-1.1018	
VII/21/72	3.1-23.4	31	18	4	0.7011	14.75**	-1.2526	
X/16/72	0.5-29.0	25	20	5	0.9297	106.48**	-1.3979	
VIII/14/73	2.4-14.2	29	24	5	0.9600	17.29*	-1.3739	
XI/15/73	2.0-28.2	21.5	12	5	0.2978	1.32	-0.8849	

* Significant at 95% level.

** Significant at 99% level.

TABLE II

Summary of analysis of covariance for testing significance of differences between slopes and intercepts for regression equations of oxygen uptake in *Mellita quinquesperforata* at different temperatures, in different nutritional states, on a dry (DW) and nitrogen (NW) weight basis, and in closed and open respirometers. The form of the linear regression equation is $\log_{10}$ ml O_2 respired/hr per animal (Y) equals $\log_{10}$ g dry body weight or nitrogen weight (X) times slope plus intercept. For comparing slopes, summer months are considered as VI, VII, and VIII; fall months are IX, X, and XI; winter months are XII, I, and II; and spring months are III, IV, and V.

Dates or experiments being tested	Pooled slope	χ^2 variance	"F" for slope	df	"F" for intercept	df
Tests for heterogeneity among experimental dates						
All non-starved dates (DW)	0.5485	13.61	0.3027	9, 36		
Summer non-starved dates (DW)	0.5374	5.10	0.1323	2, 11	21.7575**	2, 27
Fall non-starved dates (DW)	0.5614	4.20	2.2056	2, 9	0.3768	2, 23
Winter non-starved dates (DW)	0.5327	0.90	0.0827	2, 12	7.8714**	2, 29
All non-starved dates (NW)	0.5670	14.55	1.1036	8, 32	1154.6722**	8, 327
All starved dates (DW)	0.8216	2.37	1.5747	4, 14		
All starved dates (NW)	0.8132	7.77	2.5699	4, 14	15.9071**	4, 75
Tests for heterogeneity among grouped regressions						
Starved vs non-starved summer dates (DW)	0.5789	0.83	7.7766**	1, 24	2.3639	1, 25
Starved vs non-starved fall dates (DW)	0.6611	15.43**	6.2446**	1, 21	1.8405	1, 27
Starved vs non-starved summer dates (NW)	0.6359	0.01	2.1658	1, 21	0.0364	1, 22
Starved vs non-starved fall dates (NW)	0.6572	10.42**	2.9027	1, 21	0.2687	1, 22
Closed vs open at 25° C	0.6772	1.09	0.0750	1, 8	62.4133**	1, 9
Closed vs open at 20° C	0.5496	0.90	0.0436	1, 26	23.2153**	1, 27
Closed vs open at 30° C	0.4115	12.52**	0.0456	1, 20	11.8181**	1, 21

* Significant at 95% level.

** Significant at 99% level.

temperature (Table I; Fig. 2). Small animals have proportionally higher rates of respiration than do large animals when values are expressed on a dry weight (pooled yearly slope of 0.5485; Table II) or a nitrogen weight (pooled yearly slope of 0.5670; Table II) basis. Differences between slopes are not significant when slopes are compared together by year or by season (Table II). Degree of gonadal development had no effect on respiratory rates.

Although there was no significant difference between slopes, rates of respiration did change significantly with water temperature, as indicated by significant "F" values for intercepts (Table II). To simplify comparison at different temperatures, intercepts were adjusted using a pooled slope of 0.5485 (monthly slopes were not significantly different) for all experiments. Using these adjusted intercepts, a 5-g animal (dry weight) would have highest rates of respiration on VII 4/72 at 31° C (0.4259 ml O_2 /hr) and lowest rates on II 27/73 at 15° C (0.1845 ml O_2 /hr) (Fig. 2). Rates of respiration slowly decrease between 30 and 22° C (fall months) but decline greatly between 31 and 30° C (VII 4/72 and VII 25/72, respectively). The respiratory rate is lower at 33° C than at 31° C (Fig. 2). Q_{10} values for field acclimatized animals are 1.38 from the temperature range 15 to 33° C, 1.69 from 15 to 31° C, 1.93 from 22 to 31° C, 1.13 from 22 to 30° C and 1.42 from 15 to 22° C.

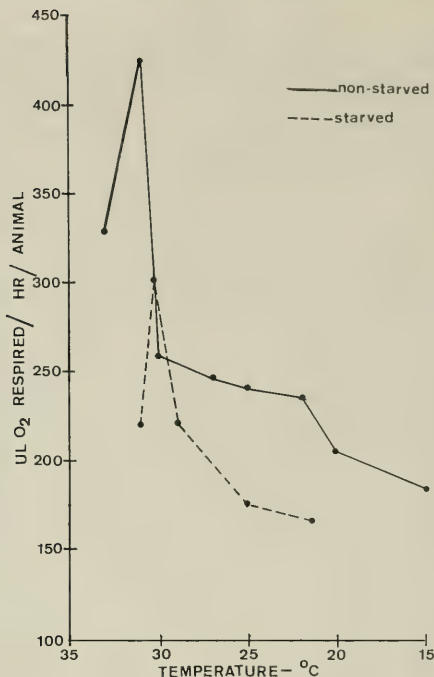


FIGURE 2. Relationship between oxygen consumption ($\mu\text{l O}_2$ respired/hr per animal) and water temperature for a starved and a non-starved 5-g (dry) specimen of *Mellita quinquiesperforata*. Rates were calculated from regression equations given in Table I.

Respiratory rates decline with starvation; the degree to which the rate declined depended on length of time animals were starved, water temperatures during starvation, and weight of the animal. Because animals were starved for different lengths of time at different water temperatures, it is difficult to assess the effect of these variables independently. Respiratory rates became more depressed as the period of starvation increased (Table I). A 1-g dry animal which was starved for 18 days at 31°C (VII/21/72) would use $53.63 \mu\text{l O}_2/\text{hr}$, whereas a 1-g animal starved for 5 days at 30°C (VII/30/72) would use $76.24 \mu\text{l O}_2/\text{hr}$ (calculated using a pooled slope of 0.8670 and adjusted intercepts, Table I).

Effects of starvation depend on weight of the animals. On the basis of body dry weight, starvation has a proportionally greater effect on the respiratory rate of small starved animals as indicated by higher values for "starved" slopes (Table I). A 20-g (dry weight) starved animal has slightly higher rates and a

5-g (dry weight) starved animal has lower rates when compared with non-starved animals of similar size (Table I; Fig. 2). On a seasonal basis, the slopes for starved and non-starved animals are significantly different (Table II).

When respiratory rates are expressed on a nitrogen basis, O_2 uptake of small, starved animals is not as depressed when compared to non-starved animals (Fig. 3) and the regression slopes and intercepts of the two groups are not significantly different (Table II). Starved animals of all sizes have significantly less nitrogen/g dry weight than non-starved animals (Lane, 1977; Fig. 4) and small animals lose (metabolize) slightly more nitrogen during starvation than do larger animals (Lane, 1977). Small animals have lower rates of respiration when starved, but they have less body nitrogen as well when compared with larger animals.

Rates of respiration depend on the type of system used for measuring respiration. Comparison of respiration in closed and open systems at the same temperature shows intercepts, hence rates, of O_2 consumption are significantly higher in the open system (Table II). Using pooled slopes and adjusted intercepts for each

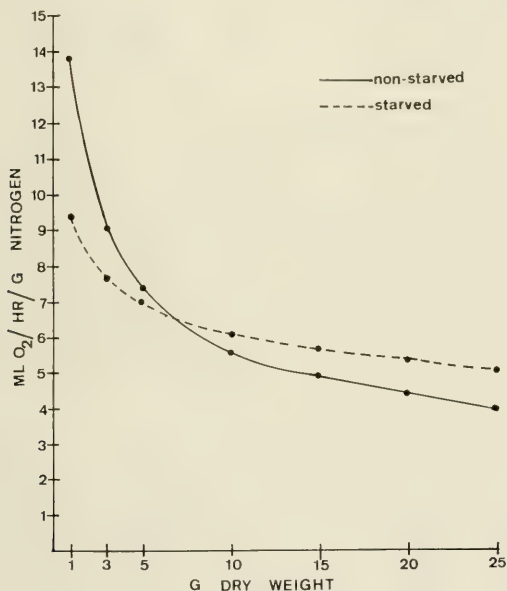


FIGURE 3. Relationship between oxygen consumption (ml O_2 respired/hr/g nitrogen) and dry body weight for starved and non-starved specimens of *Mellita quinqueperforata* at fall water temperatures. Rates were calculated as follows; non-starved animals- $\log_{10}$ ml O_2 /hr/g nitrogen equals 1.1414 minus 0.3823 times $\log_{10}$ g dry body weight; starved animals- $\log_{10}$ ml O_2 /hr/g nitrogen equals 0.9740 minus 0.1835 times $\log_{10}$ g dry body weight.

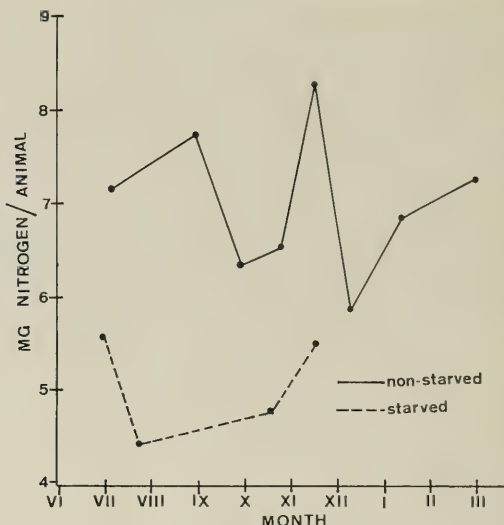


FIGURE 4. Seasonal changes in the amounts of total nitrogen in a starved and non-starved 1-g specimen of *Mellita quinquesperforata*. Values were calculated using pooled seasonal slopes and adjusted intercepts. Slopes used for non-starved animals are: summer, 0.8989; fall, 0.9462; winter, 1.0301. Slopes used for starved animals are: summer, 0.9561; fall, 1.0499.

temperature, a 10-g animal (dry weight) would respire 0.3003 ml O₂/hr per animal and 1.0598 ml O₂/hr per animal at 25° C; 0.2252 ml O₂/hr per animal and 0.5564 ml O₂/hr per animal at 20° C; and 0.4990 and 0.8158 ml O₂/hr per animal at 30° C in closed and open systems respectively. Comparison of respiration during the day and night in an open system showed no trends.

DISCUSSION

As found with many other echinoderms and invertebrates in general, the respiratory rates of *Mellita quinquesperforata* are modified by a variety of factors. Body weight or age is one such factor. Smaller (younger) animals have proportionally higher rates of respiration than larger (older) animals when rates are based on both body weight or body nitrogen. Similarly, this effect of size (age) on rate of O₂ uptake has been found in all other echinoids studied and regression slopes (log₁₀ ml O₂ respired/hr per animal versus log₁₀ body weight) for echinoids generally fall between 0.500 and 0.700 (Lewis, 1967, 1968; McPherson, 1968; Percy, 1971; Webster, 1972; Miller and Mann, 1973). An adequate or complete explanation for this phenomenon is lacking. The decreasing surface area with increasing weight explanation does not appear to apply in the case of *M. quinques-*

perforata. Surface area (from shape) is approximately body weight 0.66, while respiration is body weight 0.5485. In addition, the body wall of echinoids has been shown to be the principal consumer (90%) of oxygen in whole respiring animals (Giese, Farmanfarmaian, Hilden, and Doezenia, 1966; Webster, 1972; Belman and Giese, 1974; Webster and Giese, 1975). Stephans, Volk, Wright, and Backlund (1978) similarly concluded that the epidermis of the test of the sand dollar is responsible for a large portion of the total oxygen consumption by the sand dollar, *Dendraster excentricus*. Since the outer surface of the body wall is directly exposed to oxygenated water, the surface to volume argument should hardly apply to these echinoids.

In *M. quinqueperforata*, it would appear that smaller (younger) animals may simply have 'faster' metabolic systems, i.e., shorter half-lives of enzymes, faster turnover of proteins. Evidence for this comes from similar decreases in rate with increasing animal weight which were found for the processes of feeding, carbon excretion, ammonia excretion and radioactive uptake in this sand dollar (Lane, 1977). With starvation, smaller sand dollars metabolize proportionally greater amounts of nitrogen than larger animals. Fuji (1962, 1967) and Miller and Mann (1973) found similar allometry in different functions of other echinoids. Stephans *et al.* (1978), however, found that differences in rates of amino acid uptake between small and large sand dollars disappeared when surface area was used as the basis of expression.

As with *M. quinqueperforata*, other echinoids have depressed respiratory rates with starvation. Farmanfarmaian (1966) observed that $\dot{V}O_2$'s were reduced by as much as 50% after a month of starvation. Giese (1967) noted a general

TABLE III

Summer and fall comparisons for calories expended in respiration month of starved and non-starved *Mellita quinqueperforata*. For starved animals, values were calculated from VIII 14/72 (summer) with a slope of 0.867 and an adjusted intercept of -1.3353 and XI/15/73 (fall) with a slope of 0.7720 and an adjusted intercept of -1.3099. For non-starved animals, a slope of 0.5485 and an adjusted intercept of -0.7541 (VII/4/72) was used for calculating summer values and a slope of 0.5485 and an adjusted intercept of -1.0119 (XI/15/72) was used for fall values. 4.8 cal/ml O_2 respired was used to convert ml O_2 into calories.

Dry weight of animals	Cal respired/month/starved animal	Cal respired/month/non-starved animal	Starved values/non-starved values $\times 100$
Summer comparison			
1 g	159.67	608.80	26.22%
10 g	1175.71	2152.65	54.61%
20 g	2144.11	3148.39	68.10%
40 g	3910.80	4604.72	83.93%
Fall comparison			
1 g	169.31	336.26	50.35%
10 g	1001.55	1188.98	84.23%
20 g	1710.05	1738.95	98.33%
40 g	2919.97	2543.33	114.80%

decline in rate of oxygen uptake over a month of starvation. Webster (1972) found that the greatest decrease in V_{O_2} occurred in the first week of starvation with little change thereafter. Boolootian and Cantor (1965) reported that the respiratory rate fell to a low level after three weeks of starvation and remained constant thereafter. Differences in response of respiratory rate among echinoids with starvation may perhaps be explained by differences in nutritional history at the beginning of the experiment and consequent differences in substrate being metabolized during starvation. Wallace (1973) found that the V_{O_2} of starving crabs fell in steps corresponding to the type of nutrient reserve being metabolized. Carbohydrate reserves were first utilized, with lipid next and protein last. Differences in response of small and large specimens of *M. quinquesperforata* to starvation may also be due to differences in substrate being metabolized. If so, large animals may have had proportionally greater reserves of either carbohydrate or lipid (in the gonad). Consequently, small animals may have been metabolizing protein while larger animals were metabolizing carbohydrate or lipid, resulting in more depressed rates of respiration in small animals. The proportionally greater decrease in amount of body nitrogen in small, starved animals seems to support this conclusion.

Higher rates of respiration in small, non-starved specimens of *Mellita* may also have been due to effects of SDA or greater activity in non-starved sand dollars. Effects of both factors, however, appear to be minimal. Activity of starved and non-starved animals was similar in closed containers. As reported by Lilly (1979) for two species of sea urchins, the effect of SDA was most pronounced just after feeding and declined to pre-feeding levels after 3 hr. Rates of O_2 uptake in the sand dollar were measured approximately 24 hr after feeding. Hence SDA should be of slight consequence.

Differences between respiratory rates in starved and non-starved animals may represent energy used in growing (Table III). If so, 26% of respiration energy is used for summer maintenance and the remainder (74%) is energy expended for growth in a 1-g, non-starved specimen of *M. quinquesperforata*. In an actively growing 20-g animal (dry weight), 68% of the respiration energy is used for summer maintenance and 32% represents energy for growth (Table III). In fall, more non-starved respiration energy is used in maintenance and less in growth for all size sand dollars. Both the greater amounts of energy expended in growth by small sand dollars and the seasonal difference in amounts of energy for growth by all size animals are consistent with information from growth studies on this animal (Lane, 1977). From growth studies, a 1-g animal would expend 136 calories for growth/month in the summer (Lane, 1977). Difference between respiratory rates of starved and non-starved 1-g animals indicate 449 calories/month would be spent in growth. Likewise in fall, a 1-g animal would add 91 calories/month in growth (Lane, 1977) and expend 167 calories/month in growing (from difference between starved and non-starved respiration). For larger animals in fall, starved respiration was greater than non-starved. This anomaly may have resulted from differences in the nutritional condition of "freshly collected" animals. Negative growth reported for large animals in fall (Lane, 1977) could indicate that "freshly collected" specimens of *M. quinquesperforata* were

already starved when taken from the field, and starvation in the laboratory produced no further change in respiratory rate.

Although laboratory experiments were not specifically designed to characterize type of acclimation response in *M. quinquesperforata*, monthly oxygen measurements made at ambient water temperatures did demonstrate partial acclimatization in sand dollars. V_{O_2} 's were higher in January at 20° C than in December at the same temperature. Likewise, acclimatization to summer water conditions may have resulted in lower rates of respiration on VII/25/72 at 30° C as compared to the much higher rates three weeks earlier on VII.4/72 at 31° C. As with many other echinoids (Farmanfarmaian and Giese, 1963; Moore and McPherson, 1965; McPherson, 1968; Percy, 1971; Webster, 1972), this compensatory response was only partial in *Mellita* since respiration was still higher in summer than in winter.

The sensitivity of the respiratory rate in *M. quinquesperforata* (Q_{10}) was relatively low between 22 and 30° C. In the Tampa Bay area, the change in water temperature over the 22 to 30° C range is rapid during the spring warming and fall cooling. The respiratory insensitivity over this range would, therefore, appear to have some adaptive value. The decline in V_{O_2} at 33° C may indicate metabolic malfunction as this temperature is close to the lethal limits (~38° C) of this sand dollar.

Oxygen consumption may be modified by type of system used for measuring respiration. Miller and Mann (1973) for *S. droebachiensis*, Webster and Giese (1975) for *Strongylocentrotus purpuratus* and this study found that rates of respiration were higher in flow-through systems when compared with closed systems. Although this increased rate may be caused by increased availability of oxygen, activity of animals may also be increased in flowing water. Activity of *M. quinquesperforata* was much greater in the flow-through system than intermittent activity of sand dollars in closed systems. Although sand dollars are exposed to a continual exchange of water in their environment, much of the time they are partially buried and do not or can not continually wave their spines back and forth as was the case in the open system. Therefore, the monthly oxygen measurements made in closed dishes are considered more representative of routine metabolism.

Due to various factors which modify metabolic rate, and the different units used to express respiratory rate, it is difficult to compare absolute rates of respiration among various echinoids. When compared to the respiratory rates of tropical and temperate echinoids as given by Webster (1975), V_{O_2} 's of *M. quinquesperforata* (as found in this study) are slightly higher than rates for tropical echinoids of similar weight.

SUMMARY

1. Rates of respiration in a closed vessel conformed to oxygen concentrations in surrounding water until a low level (1 ml O_2 /liter) of oxygen was attained.

2. Respiratory rates of small animals were proportionally higher on both a dry weight and nitrogen weight basis than rates of large animals (slope for ml O_2 respired/hr per animal was 0.5485 for dry weight and 0.5670 for nitrogen) in a closed vessel.

3. Respiratory rates of field acclimatized animals were slightly higher at summer temperatures (30–33° C) than at winter water temperatures (15–20° C) with evidence for partial acclimatization presented.

4. Respiratory rates of starved animals were lower than rates of non-starved animals with starvation depressing the rates of smaller animals more than rates of larger animals.

5. Respiratory rates in an open system were approximately twice as high as rates in a closed system due to greater activity of animals in the open system.

6. Comparison of day versus night respiration showed no obvious trends.

7. Respiratory rates of *M. quinqueperforata* are slightly higher than rates of a similar weight, tropical, regular urchin as reported in the literature.

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DEVELOPMENT AND BEHAVIOR OF AN INTERGENERIC CHIMERA
OF HYDRA (*PELMATOHYDRA OLIGACTIS* INTERSTITIAL
CELLS: *HYDRA ATTENUATA* EPITHELIAL CELLS)

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Hydra has so few cell types that it should be possible to map out the developmental and behavioral functions of each. By combining cell types of different species, one might trace roles by identifying species characters in the resulting chimeric animals.

Many of hydra's highly specialized cell types (nerve cells, nematocytes, gametes) are part of a single lineage of cells that is continually being renewed by proliferation and differentiation of a stem cell called the interstitial cell or "I cell." The entire interstitial cell lineage can be removed from a hydra by various means (Marcum and Campbell, 1978a; Campbell, 1979). The resulting animal is termed an "epithelial hydra," and is composed only of ectodermal and endodermal epithelial cells. This viable epithelial shell can then be repopulated by I cells, since they will migrate throughout the depleted animal from a small, temporary graft of normal tissue. A number of chimeric strains in hydra have been made in this fashion (Saffitz, Burnett and Lesh, 1972; Sugiyama and Fujisawa, 1978; Marcum and Campbell, 1978b). In order to assign roles to the different cell lineages, one would use the most dissimilar species as parents. However, grafting success (and hence presumably tissue compatibility) decreases as species diversity increases (Campbell and Bibb, 1970), so that most hydra chimeras have been constructed of cells from the same or closely related species.

One pair of dissimilar species, *Hydra attenuata* and *Pelmatohydra oligactis*, will partially tolerate intergrafting and a considerable literature suggests that it may be possible to make a stable chimera between their cells (Evlakowa, 1946; Brien and Reniers-Decoen, 1955; Kolenkine and Bonnefoy, 1976). Since it is possible to remove the interstitial cell lineage from *H. attenuata*, we repopulated epithelial *H. attenuata* with *P. oligactis* interstitial cells. The reciprocal graft is not possible since a technique for removing I cells from *P. oligactis* has not been found. This report describes some developmental and behavioral similarities and differences between the chimeras and the two parental species.

MATERIAL AND METHODS

Specimens of *Hydra attenuata* from Lake Zurich, specimens of *Pelmatohydra oligactis* collected in Grant Lake, Mono County, California, and the chimeras were all grown in "M" solution lacking bicarbonate (Muscatine and Lenhoff, 1965) by standard methods (Lenhoff and Brown, 1970). Epithelial specimens of *H. attenuata* were produced by a double colchicine treatment (Marcum and Campbell,

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1978a) to eliminate I cells. Distal halves of epithelial specimens of *H. attenuata* were repopulated with I cells by axially grafting them to proximal halves of normal *P. oligactis* polyps. The graft junction was marked by a permanent constriction in the ectoderm (Kolenkine and Bonnefoy, 1976) and by different coloration of the endoderm in these two species. Grafts were left intact for 3 to 5 days and then the *P. oligactis* epithelial tissue was cut away. The resulting repopulated hydra were maintained for up to 6 months by methods appropriate for epithelial hydra (Marcum and Campbell, 1978a).

Cell compositions were determined using David's (1973) maceration procedure. Heat shocks were applied by immersing 10-ml test tubes containing individual hydra in 2 ml of medium into a preheated waterbath for 30 min. Afterwards the tubes were left at room temperature for 12 hr, and then the hydra were cultured normally. Time-lapse films were made using a 16-mm Bolex camera outfitted with extension tubes behind the lens, with illumination through heat filters.

Feeding response was measured using the methods of Lenhoff (1969). Inhibition of nematocyst discharge (Smith, Oshida and Bode, 1974) was carried out by feeding hydra to repletion and then releasing single shrimp, successively, onto tentacles at measured times later. The number of shrimp contacts which occurred before two became trapped was recorded. Only 20 trials were offered each polyp; a score of 20 indicated that the hydra did not catch the *Artemia*.

RESULTS

Genetic composition of chimeras

Although the methods used for producing chimeras seem straightforward, we considered it important to demonstrate that the chimeras were, in fact, composed of *H. attenuata* epithelial and *P. oligactis* interstitial cells.

The genetic origin of interstitial cells was verified by analysis of chimera nematocysts. Table I shows the dimensions and Figure 1 shows the morphologies

TABLE I

Nematocyst sizes. Length and width (standard deviations in parenthesis) are all in μm . Each measurement represents about 20 nematocysts. Chimera polyps a, b, c, and d are progeny from different grafts.

Hydra strain	Stenotele	Atrichous isorhiza	Desmoneme
<i>H. attenuata</i>	14.8×11.4 (2.2) (1.9)	9.7×4.0 (1.0) (0.2)	8.1×5.6 (0.6) (0.4)
<i>P. oligactis</i>	12.9×9.7 (0.5) (0.5)	7.8×4.3 (0.5) (0.5)	6.7×4.8 (0.4) (0.3)
Chimera polyp a	12.1×9.5 (0.7) (0.7)	8.2×4.0 (0.7) (0.2)	5.7×4.2 (0.5) (0.4)
polyp b	11.9×9.1 (0.6) (0.8)	8.8×4.4 (0.5) (0.6)	6.2×4.6 (0.4) (0.4)
polyp c	11.0×8.3 (1.0) (0.7)	8.8×3.8 (0.6) (0.3)	6.1×4.0 (0.5) (0.1)
polyp d	11.7×8.9 (0.6) (0.5)	9.1×4.0 (0.8) (0)	6.1×4.2 (0.2) (0.2)

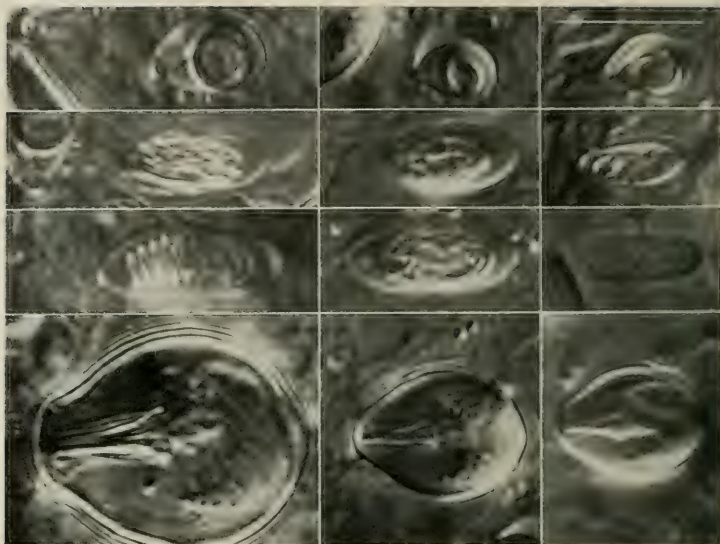


FIGURE 1. Nematocyst structures of *H. attenuata* (left), *P. oligactis* (middle), and chimera (right). Nematocyst types, bottom to top, are: stenotele, holotrichous isorhiza, atrichous isorhiza, and desmoneme. All figures except the chimera holotrichous isorhiza represent mature, mounted nematocysts. Chimeras rarely have mounted holotrichous isorhizas, but the species specific tubule pattern is visible in complete but immature capsules in gastric region nests, as shown here. Scale indicates 10 μ m.

of nematocysts of the chimeras and both parents. These data are taken from animals more than 4 months after chimeras were made. The small sizes of the chimera nematocysts are similar to those of *P. oligactis* and not *H. attenuata*. The morphologies of the chimera nematocysts (Fig. 1) are also unambiguously those of *P. oligactis*. The absence of transverse coils in the holotrichous isorhiza is the

TABLE II

Relative proportions of nematocyst types in tentacles. Numbers express percentages of total nematocysts. (Between 500 and 1000 nematocysts were counted in the distal parts of tentacles, with the number of polyps indicated at left).

Species	Nematocyst type		
	Stenotele	Isorhizas	Desmoneme
<i>H. attenuata</i> (n = 2)	3.5 (± 0.7)	12.5 (± 4.9)	84 (± 4)
<i>P. oligactis</i> (n = 3)	34 (± 9)	5.7 (± 1.5)	60 (± 9)
Chimera (n = 6)	37 (± 8)	6.7 (± 1.0)	57 (± 7)

most notable character of *P. oligactis* stingers (Ewer, 1948), and the chimera also lacks these. In addition, the slender shape of the stenotele, the bluntly oval shape of the holotrichous isorhiza, and the reniform atrichous isorhiza are all characteristically *P. oligactis*.

The two parental species differ in the relative abundances of the different nematocyst types. Table II shows that in this character the chimeras closely resemble *P. oligactis* and not *H. attenuata*. However, one abnormality of the chimeras was the nearly complete absence of mature holotrichous isorhizas. None were found on animals whose nematocytes were measured (Table I), and few were seen in this study. The photograph in Figure 1 is of an immature holotrichous isorhiza.

The genetic origin of epithelial cells was ascertained in two ways. First, in color both the ectoderm and endoderm were found to resemble *H. attenuata* rather than *P. oligactis*. The chimeric ectoderm was colorless, and the endoderm pink, as in *H. attenuata*. *P. oligactis* has yellow granules in the ectoderm and an orange endoderm. Second, it is known that epithelial tissue controls graft tolerance (Campbell and Bibb, 1970). Therefore, two chimeras that had been established for 6 months were bisected and halves were grafted back to the two parental strains. In the chimera *H. attenuata* grafts, the graft junctions became imperceptible within a day and no incompatibility was detected during the next 8 days of culture. In the chimera/*P. oligactis* grafts, the graft junctions were still constricted after 1 day and by the sixth day the two halves had separated.

We conclude that the chimeras had *H. attenuata* epithelial cells and *P. oligactis* interstitial cell lineage, and that this composition remained stable throughout the study.

Morphology of chimeras

Chimeras were always smaller than either parent (Fig. 2). Measurements of ten chimeras and parents grown on a regime of six shrimps day averaged 2.0 mm (chimera), 7.0 mm (*H. attenuata*) and 9.0 mm (*P. oligactis*) in extended length. Tentacle number of budding individuals averaged 5.8 (chimeras), 6.5 (*H. attenuata*) and 6.4 (*P. oligactis*) per polyp. The body column and tentacles of chimeras never seemed to elongate as much as those of either parental species, and this was a contributing factor to their short lengths. The chimeras were slightly more stalked than the *H. attenuata* parent, but not as pronouncedly so as the *P. oligactis* parent.

Budding

The most clear-cut difference between buds of the parental species is the arrangement of tentacle rudiments. Buds of *P. oligactis* first acquire two lateral tentacles, and after these have grown long, two more intercalated rudiments arise (Fig. 3a). In *H. attenuata* the tentacle rudiments arise nearly synchronously and are all about the same length (Fig. 3c). The chimeric pattern (Fig. 3b) is clearly of the *H. attenuata* type.

The budding rates of chimeras were about normal. In one experiment three polyps each of *H. attenuata*, *P. oligactis* and chimera were fed six shrimps/day,

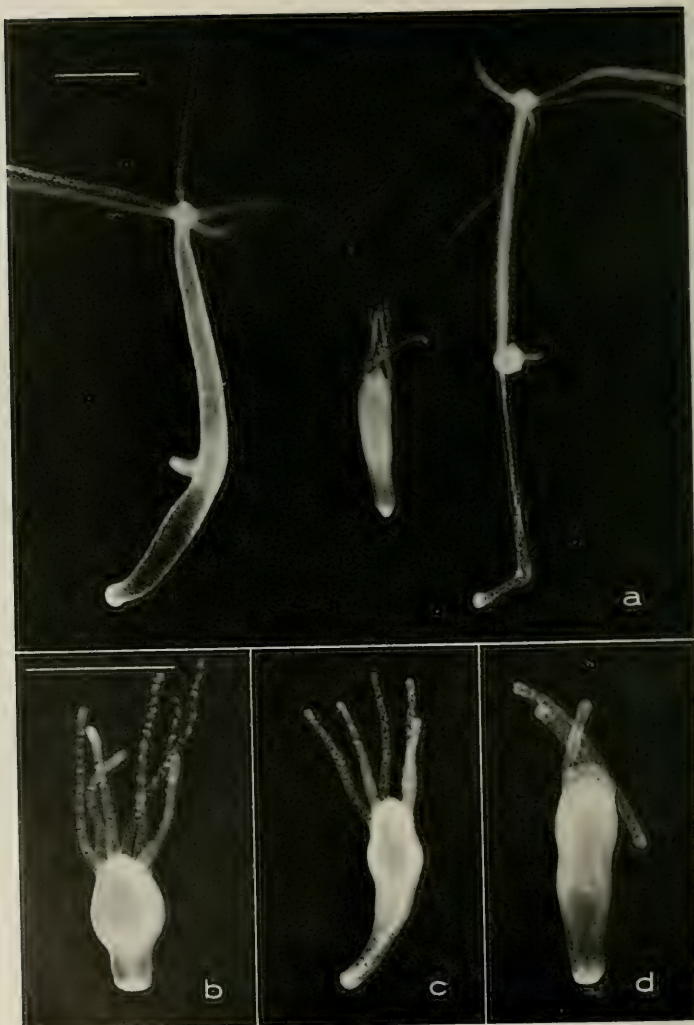


FIGURE 2. Morphology of hydra. (a) *H. attenuata* (left), chimera (middle) and *P. oligactis* (right). All three hydra are of the same age, growing under the same conditions. (b)-(d) different chimera individuals showing typical poses. Scale indicates 1 mm.

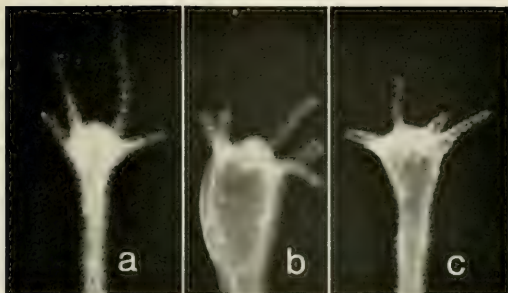


FIGURE 3. Pattern of tentacle development on buds. (a) *P. oligactis* (b) chimera, (c) *H. attenuata*.

long enough for each to produce five buds. Budding rates for these three strains were, respectively, 0.26, 0.26, and 0.20 buds/day. Early morphogenesis, until basal disk formation, of the developing buds occurred at normal rates. However, chimera buds remained attached to their parents for an average of 8 days, while both parental buds detached after 3 days. Mature chimera buds were very small, averaging 1.2 mm in length (extended), whereas *H. attenuata* buds average 5.1 mm and *P. oligactis* buds averaging 5.4 mm in length. Chimera buds did not themselves begin to bud for 19 days, while parental buds budded after 10 (*H. attenuata*) or 11 (*P. oligactis*) days.

Regeneration

Polyps that had been fed six shrimps/day for 6 days were cut in half transversely and both parts allowed to regenerate, without feeding. Tentacle regeneration was assayed by the presence of tentacle rudiments. Basal disk regeneration was assayed by adherence to the dish.

The chimeras ($n = 3$) and both parental species (*H. attenuata*, $n = 8$; *P. oligactis*, $n = 7$) regenerated tentacles from the proximal half in 2 days. The distal halves of chimeras and of *H. attenuata* regenerated basal disks in 2 days. However, the *P. oligactis* proximal halves had not regenerated basal disks in 26 days. Thus, in basal disk regeneration the chimera resembled the epithelial cell parent.

Feeding behavior

The chimeras (as well as the two parental strains) showed typical (Lenhoff, 1969) feeding responses to *Artemia*. When shrimp touched the tentacles they adhered, indicating desmoneme nematocyte discharge, and were paralyzed, indicating stenotele nematocyte discharge. Tentacles holding shrimp underwent considerable writhing, and shrimp were brought to the mouth repeatedly. However, in the chimera the shrimp were never swallowed. The chimeras were thus unable to feed themselves.

To determine if the swallowing behavior itself was deficient in the chimeras,

TABLE III

Inhibition of nematocyst discharge following satiation. Numbers represent average number of trials before hydra caught and paralyzed two Artemia. Twenty was the maximum number of trials allowed for any polyp. These data represent about 100 sets of trials.

Time after feeding (min.)	Hydra strain		
	<i>H. attenuata</i>	<i>P. oligactis</i>	Chimera
10-30	5.8	7.5	5.4
30-60	12	16	8.4
60-120	10	20	6.7

we strung isolated hydranths on nylon fishing line in "M" solution and measured the creeping movement along the line. In normal hydra the hydranth will move rapidly along the line in the direction of the mouth, as the hydranth attempts to swallow the line. After the first 20 min, *H. attenuata* ($n = 1$) had moved 2.4 mm and *P. oligactis* ($n = 1$) had moved 0.9 mm. Two chimera hydranths failed to move during 3.5 hr of observation.

Normal hydra exhibit a mouth opening response to glutathione. The duration of opening reflects the intensity of the response (Lenhoff, 1969). The durations of the feeding responses to 10^{-5} M glutathione (26° C, pH 7.0) were measured with the following results: *H. attenuata* ($n = 10$), 35.2 ± 18.6 min; *P. oligactis* ($n = 11$), 41.9 ± 13.5 min; chimera ($n = 26$), 21.7 ± 6.8 min.

We also tested the satiation response, as manifested by the failure of nematocysts to discharge, and consequently the failure to trap *Artemia*, after the hydra is fed (Smith, Oshida and Bode, 1974). Table III shows data from these experiments. The chimeras showed a reduced satiation response up to two hours after feeding.

Body motility

Chimeras were much less active than were the two parents. Chimeras never somersaulted nor extended fully, while parental polyps frequently did. We analyzed body contractions and pulsations using time-lapse motion pictures showing all three polyp types in the same dish. By "contractions" we refer to a marked shortening of the body column followed by a reextension. "Pulsation" refers to peristaltic waves traveling proximally down the column.

In contraction frequency, chimeras (0.25/min) were intermediate between *H. attenuata* (0.08/min) and *P. oligactis* (0.34/min). In pulsation frequency chimeras (1.07/min) resembled *H. attenuata* (1.03/min). *P. oligactis* did not exhibit pulsations.

DISCUSSION

The major objective of this research was to produce a chimeric strain of hydra in order to distinguish developmental and behavioral contributions of the epithelial and interstitial cell lineages. Other studies of this nature (Sugiyama and Fujisawa, 1978; Marcum and Campbell, 1978b) showed the feasibility of this approach using closely related strains or species. In the present study we sought to examine the

feasibility of using distantly related species, in this case species of separate genera.

In several traits it was possible to attribute chimeric development and behavior to particular cell types. The pattern of tentacle origin on buds, the rate of basal disk regeneration, heterospecific grafting specificity, color, and prominence of columnar peristaltic waves were all clearly characteristic of *H. attenuata*. Thus, these traits are determined by epithelial cells. On the other hand, nematocyst morphology, nematocyst concentration, and interstitial cell temperature sensitivity (Fradkin, Lee, and Campbell, unpublished) were distinctively those of the *P. oligactis*, indicating that these characteristics are due to the interstitial cell lineage. These results fit the pattern so far uncovered (Campbell, 1979) that morphological and morphogenetic traits derive principally from the epithelial cell genotype.

In several respects the evolutionary divergence between *H. attenuata* and *P. oligactis* was apparently too great to allow normal chimera functioning. These chimeras were very delicate, and had such altered behavior that they could neither eat nor somersault by themselves. Repopulation of epithelial hydra by interstitial cells of the same species yields hydra of normal behavior (Marcum and Campbell, 1978b) suggesting that the behavioral defects observed here are due to cell incompatibilities rather than due to the repopulation procedure. Therefore, in constructing chimeras for purposes of deducing cellular roles one must work with more closely related species. However, chimeras containing cells as divergent as *H. attenuata* and *P. oligactis* certainly may be useful in unravelling cellular mechanisms. It would be interesting, for example, to see if abnormal neuromuscular contacts might be responsible for the inadequacy of the feeding behavior or the absence of column elongation.

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SUMMARY

An intergeneric chimera was produced by repopulating epithelial *Hydra attenuata* (lacking the interstitial cell lineage) with interstitial cells of *Pelmatohydra oligactis*. The chimera's morphology and morphogenesis generally resembled that of *H. attenuata*, for example in the pattern of bud tentacles, in basal disk regeneration rate, and in heterografting specificity. Nematocyst characters of the chimera were the *P. oligactis* type. In behavior the chimeras were intermediate in some respects but deficient in others. For example, chimeras were unable to feed by themselves or to extend the column. This study illustrates the value of chimeras in deducing which cell types control the various aspects of development and behavior.

NOTE ADDED IN PROOF

Data from cell type composition studies show that the percentage of nerves among the total cells counted for *H. attenuata* is 5.5%, for *P. oligactis*, 5.7%, and for the Chimera, 6.4%. A total of 5000 to 9000 cells were counted for 6 to 9 individuals (6 individuals of *H. attenuata*, 6 of *P. oligactis*, and 9 of Chimera).

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ON THE POPULATION BIOLOGY AND NATURE OF DIAPAUSE
OF *LABIDOCERA AESTIVA* (COPEPODA: CALANOIDA)¹

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Knowledge of the seasonal distribution patterns of many marine copepods led Fish and Johnson (1937) to postulate that these species might produce resting eggs as an adaptive mechanism to insure survival at times of the year unfavorable to a planktonic existence. It was not until 1972 (Zillioux and Gonzalez, 1972) that conclusive results demonstrating the production of resting eggs by a marine copepod were obtained. During the last decade, it has become increasingly evident that the production of resting eggs by temperate, inshore marine copepods is a widespread event in this group (Zillioux and Gonzalez, 1972; Kasahara, Onbe and Kamigaki, 1975; Landry, 1975a; Grice and Gibson, 1975, 1977; Grice and Lawson, 1976; Uye and Fleminger, 1976). These studies have documented the existence of resting eggs in bottom sediments (Kasahara, Uye and Onbe, 1974, 1975; Kasahara, Onbe, and Kamigaki, 1975; Grice and Gibson, 1975, 1977; Uye and Fleminger, 1976) and demonstrated their production by females collected from the plankton (Grice and Gibson, 1975, 1977; Grice and Lawson, 1976; Uye and Fleminger, 1976; Zillioux and Gonzalez, 1972). These investigations have shown that physical factors such as temperature, light, salinity, and oxygen concentration affect the maintenance and termination of the resting egg. The events which actually trigger the induction of resting egg production are still poorly understood. The elucidation of factors which influence the induction, maintenance, and termination of copepod resting eggs is necessary to achieve a better understanding of the biology, evolution and distribution patterns, both in time and space, of neretic temperate-boreal copepods which are key elements in the food web.

Labidocera aestiva is a large calanoid copepod reported to occur in coastal waters from the Gulf of St. Lawrence to the Gulf of Mexico (Wheeler, 1901; Grice, 1956; Fleminger, 1957; Cronin, Daiber and Hulbert, 1962; Anraku, 1964; Van Engel and Tan, 1965; Bowman, 1971; Fleminger, 1975). The populations occurring north of Cape Hatteras are seasonally abundant in the plankton, with maximum numbers of nauplii, copepodites, and adults occurring in the summer and fall, and disappearing by mid-December. Grice and Gibson (1975) and Grice and Lawson (1976) have demonstrated the production of resting eggs by females of *L. aestiva* collected in the field and their presence in bottom sediments.

During the summer-fall period in 1977, a preliminary investigation of subitaneous and resting egg production by *L. aestiva* from Vineyard Sound, Massachusetts was conducted. This work indicated that the production of resting eggs commenced in early September. The surface water temperature was 19.5° C. The data presented in this paper document the seasonal variation in subitaneous and resting

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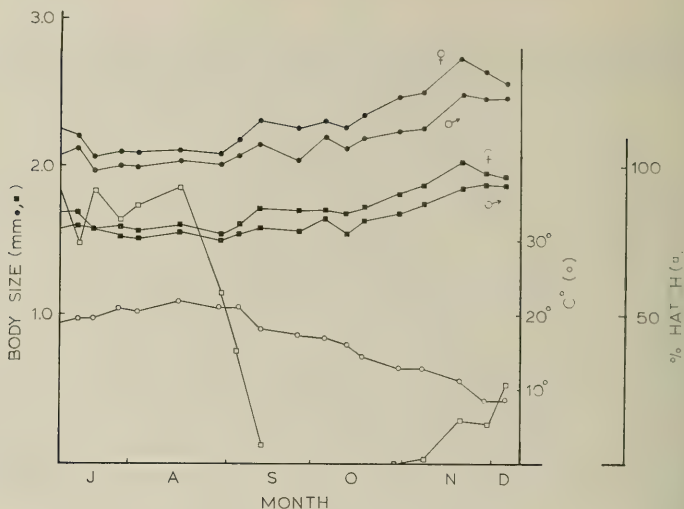


FIGURE 1. Trends in body size (cephalothorax, solid square, total length solid circle), percent hatch (open square), and surface water temperature (open circle) for the sampling period July 7, 1978 to December 6, 1978.

egg production during the summer-fall period in 1978. It is shown that the proportion of subitaneous eggs produced is greater during the summer, decreases markedly in September, and persists as a small percent throughout the fall. Moreover, the major change from subitaneous to resting egg production precedes the initiation of increasing adult body size and decreasing surface water temperatures by 2 weeks. It is demonstrated that resting eggs which are chilled at 5° C for a minimum period of 30 days, will hatch synchronously within 2 days when warmed to 21° to 23° C. Comparable eggs kept continuously at 19° to 23° C will hatch over a longer period of time and not synchronously. This paper discusses the significance of these findings as they relate to diapause induction, maintenance, and termination, as well as the population dynamics of *L. aestiva*.

MATERIALS AND METHODS

Specimens of *Labidocera aestiva* were collected from July through December at weekly intervals from a 1 to 2 m depth in Vineyard Sound, Massachusetts by towing a 1-m, 243- μ mesh net for a 10 min period. Adult males and females were removed from these two samples by pipette. Twenty individuals of each sex were preserved in 5% buffered formalin each week, except for collections in late fall when very few adults were present. Two measurements of body size were obtained for each

preserved specimen (*i.e.*, cephalothorax, and total length) using a stereomicroscope and ocular micrometer. During the summer months, eggs were collected by placing 10 females together in separate 100-ml dishes. *Gymnodinium nelsoni* was added as food, and the dishes were placed overnight in an incubator at 19° C, with a 12 to 12-hr photoperiod. The following morning eggs were removed by pipette and counted. The percent hatch was determined for an aliquot (100–120) of these eggs. During the summer, eggs usually hatched within 2 days. The same procedure of collection, counting, and determination of percent hatch was followed for fall eggs. Fall eggs which did not hatch within 4 days were placed in filtered sea water (glass fiber) in 75-ml screw-top glass jars. The jars were incubated at 19° C or refrigerated at 5° C. At the appropriate time, the refrigerated jars were warmed to 21° to 23° C and percent hatch determined. At the same time, the percent hatch in the jars kept at 19° C was determined.

RESULTS

The trends in body size, percent immediate hatch, and surface water temperature for the six-month period between July and December 1978 are shown in Figure 1. The standard deviations for body size ranged between 0.01 and 0.17. For each sex, changes in cephalothorax length paralleled total length. At each census throughout the sampling period, female body size was larger than male body size. An inverse relationship was observed for body size and surface water temperature for both females and males, and is depicted for adult females in Figure 2a. The smallest adults were collected during July and August when water temperatures ranged between 20° to 22° C. A marked increase in size began in September reaching a maximum in late November.

The percent immediate hatch (*i.e.*, within 2–4 days) of eggs collected from grouped females was greatest from July to mid-August, ranging between 74 and 93%. Subsequently the percent immediate hatch began to decrease and was observed to vary between 6 and 27% from mid-September to early December. The marked decrease in the portion of eggs hatching immediately began in late August, and preceded the initial drop of surface water temperatures to below 20° C (Figs. 1, 2b) and the time at which body size began to increase. No obvious difference in total egg production was observed, although differences may exist.

The percent hatch of eggs incubated at 19° or 5° C and then later warmed to room temperature (21°–23° C) is shown in Table I. The data presented are for eggs laid within 1 day of collection for five samplings between early September and late November. In general the percent hatch of eggs kept at 5° C and then warmed to 21° to 23° C was higher than those incubated at 19° C. Moreover, the hatch of the 5° C eggs occurred synchronously within two days of being warmed to 21° to 23° C. It was determined from periodic examination of the jars held at 19° C that non-synchronous hatching occurred throughout the incubation interval. Some of the unhatched eggs at 19° C appeared dead (brown, granulated interior), whereas others appeared viable (greenish hue).

DISCUSSION

An inverse relationship between body size and temperature has been demonstrated for a number of marine and freshwater copepods (Coker, 1933; Marshall,

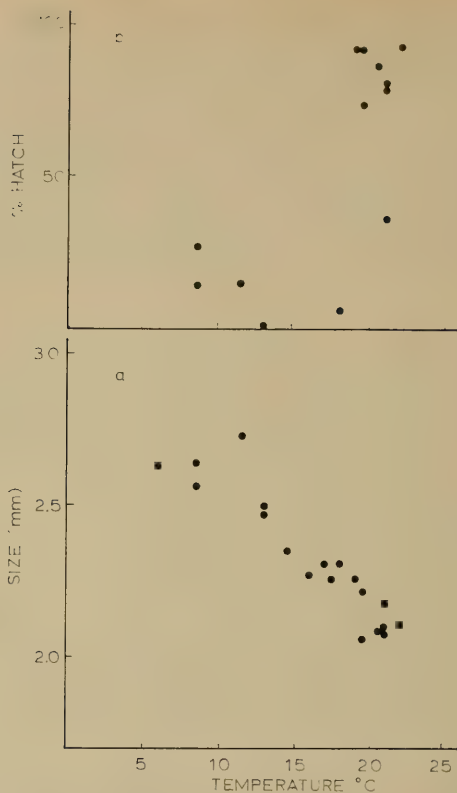


FIGURE 2. Scatter diagrams of female total body length (a), and percent hatch (b) versus surface water temperature at the time of collection.

Nicholls, and Orr, 1934; Aycock, 1942; Deevey, 1960a, b; McLaren, 1965; Deevey, 1966; see review in Hutchinson, 1967; Durbin and Durbin, 1978). The seasonal pattern of size variation reported herein for *Labidocera aestiva* from Vineyard Sound is similar to that reported by Deevey (1960a) for another population of the species from Delaware Bay studied in 1930. However, the patterns are shifted in time. The smallest individuals are found during August in Vineyard Sound and September in Delaware Bay. Deevey (1960a) reports that the average surface water temperature in Delaware Bay was 22° C during September. This value

TABLE I

Percent hatch of incubated (19°-23° C) and refrigerated Clutch 1 eggs after warming to 21°-23° C, for five samplings between early September and late November.

Date collected	Date incubated		Period of incubation or refrigeration (days)	% Hatch
	5°	19-23°		
Sep 27	Oct 2		75	96
Sep 27		Oct 2	75	61
Oct 5	Oct 10		111	95
Oct 5		Oct 10	111	42
Sep 7	Sep 11		58	83
Sep 7		Sep 11	58	2
Oct 19	Oct 23		126	95
Oct 19		Oct 23	126	55
Nov 21	Nov 27		87	95
Nov 21		Nov 27	87	95

corresponds to temperatures recorded for Vineyard Sound in August (Fig. 1). The pattern, however, varies from year to year within an area, inasmuch as the smallest individuals during 1932 in Delaware Bay were collected in early August, when temperatures were then highest. It is obvious that body size is correlated more with the surface water temperature than with the time of collection.

In the present study, there was no evidence of discrete generations for *L. aestiva* in Vineyard Sound. Breeding appeared to be continuous throughout the summer and fall. Deevey (1960a) suggested that spawning was continuous during the summer for the Delaware Bay population, but that a break occurred between early August and September, resulting in a burst of larger individuals late in October. Moreover, it was suggested that the offspring of the large-sized fall individuals would not mature until the following year. More recent studies of *L. aestiva* from Vineyard Sound (Grice and Gibson, 1975; Grice and Lawson, 1976) have demonstrated that the offspring of the fall individuals survive the winter as resting eggs, and hatch the following spring. It is probable that the same life history pattern is common to the Delaware Bay population.

In Vineyard Sound, the population continues to breed at temperatures as low as 8.5° C (Fig. 1), producing both subitaneous and resting eggs. On the other hand, Deevey (1960a) reports that 12° C is the lower limit of breeding in Delaware Bay. This difference could reflect acclimatization or genetic adaptation of individuals to the slightly differing thermal regimes of Vineyard Sound and Delaware Bay (see Bumpus, 1957). It has been shown for a wide variety of marine invertebrates that the tolerance limits for a number of biological functions differ between populations exposed to different environmental regimes (see Battaglia and Beardmore, 1978).

Whatever the factor(s) inducing the production of resting eggs by *L. aestiva* from Vineyard Sound, it is shown that the response (*i.e.* initiation of dormancy) is first expressed in late August or early September. The intensity of the response increases as time progresses and the surface water temperatures decrease. This intensification is expressed as an increase in the percent production of resting

eggs and a decrease in subitaneous eggs. There is a strong correlation between temperature and the type of egg produced (Fig. 2b). However, temperature alone is not sufficient to account for the observed switch in egg production in early September (Fig. 1). The same temperatures (*i.e.* 18°–20° C) which are coincident with the production of resting eggs in September are observed in early summer when production is restricted to subitaneous eggs. Temperature may influence the type of egg produced, but it would appear that other factors are also involved.

The data show that subitaneous eggs are produced throughout the summer-fall period, and that resting eggs are produced only during the fall. There are clearly two egg types being produced in the fall. Subitaneous eggs produced at this time take longer to hatch (2–4 days) at 21° to 23° C than those produced in the summer (1–2 days).

The rate of embryonic development of copepod eggs is influenced by egg size, temperature, and environmental conditions experienced by the parents (McLaren, 1965; 1966; McLaren, Corkett, and Zillioux, 1969; Landry, 1975b; Hart and McLaren, 1978). For *L. aestiva* the observed difference in rate of development most probably reflects a maternal effect relating to the physiological condition of the female at the time of collection, as was shown for *Pseudocalanus* by Hart and McLaren (1978). No obvious size difference was observed between summer and fall subitaneous eggs of *L. aestiva* in the present study.

The fall subitaneous eggs of *L. aestiva* were detected by placing females collected at that time in an incubator at 19° C. If the subitaneous eggs produced by fall females were collected and held at temperatures equivalent to the ambient values in Vineyard Sound, their development would be retarded and they could be mistaken for resting eggs. On the other hand, fall eggs which do not hatch within 2 to 4 days at 21° to 23° C are to be regarded as resting eggs. As first demonstrated by Grice and Gibson (1975) resting eggs of *L. aestiva* remain viable for as long as 120 days at 5° C. It was suggested that a minimum chilling period of 2 to 4 weeks at 5° C was required to break the dormant condition. The results of the present study show that if resting eggs are placed at 19° C without chilling, hatching will take place, but it takes longer and occurs sporadically. Chilling of resting eggs results in the reduction of time in actual diapause and synchronization of hatching. The diapause and hatching response of resting eggs of *L. aestiva* to different temperatures corresponds to several examples of insect and freshwater copepod diapause (Church and Salt, 1952; see Hutchinson, 1967; Stross, 1969b; Dean and Hartley, 1977a, b). The dormant state in these animals consists of a period of diapause followed by post-diapause development and then hatching (see Mansingh, 1971). During the diapause period, development is arrested and cannot resume even if conditions are favorable. Once the diapause is broken the individual is competent to resume development as soon as adverse conditions are terminated. For *L. aestiva* the actual breaking of diapause (re-activation) can take place at 19° C, but the process is faster at colder temperatures. Post-diapause development, however, is slower at reduced temperatures than at high temperatures. Therefore, individual chilled eggs terminate diapause at different times, and further development is retarded as long as the eggs remain chilled. The chilled eggs accumulate at a stage of readiness, and when exposed to higher tempera-

tures are competent to proceed with development, resulting in synchronous hatching. On the other hand, individual resting eggs held continuously at 19° C terminate the diapause condition at different times (as the chilled eggs), but since the higher temperature is favorable for post-diapause development to proceed there is no accumulation of individuals at the boundary between diapause and post-diapause so that hatching is asynchronous.

Two attributes expressed by resting eggs are cold-hardiness and synchronous hatching. Resting eggs remain viable at 2° C for as long as 6 months (Grice and Gibson, 1975) whereas summer subitaneous eggs remain viable for no longer than 15 days (Grice, unpublished). The tolerance of fall subitaneous eggs has not been investigated. The cold resistance of resting eggs enables survival during the winter. Synchronization of development promotes the reproductive success of the first generation appearing in the early summer by ensuring that individuals will attain reproductive maturity at the same time. If hatching was sporadic, the number of mature individuals in the population at any one time might not attain a size sufficient for successful mating encounters to occur.

The effects of temperature on the maintenance and termination of resting eggs of *L. aestiva* are similar to those observed for other plants and animals which overwinter. In the majority of cases for which diapause induction has also been investigated, photoperiod and temperature have been shown to be the two most important factors affecting the initiation of dormancy (Harvey, 1957; Stross and Hill, 1965; see Hutchinson, 1967; Stross, 1969a, b; Watson and Smallman, 1971; see Mansingh, 1971; de March, 1977; see Clutter, 1978). The pattern of egg production observed in this study would occur if a developmental stage perceived a cue which then triggered an irreversible sequence of events leading to one type of egg or another. The fact that resting egg production precedes the decline of surface water temperatures suggests that such a stimulus may exist. The successful species will evolve a dormancy response to a factor which closely parallels the stress (in this case temperature) but which itself is extremely stable (such as photoperiod). That two egg types are produced during the fall could be due to the extended survival of summer females which lay subitaneous eggs and the newly developed fall females programmed to produce resting eggs. Moreover, because no two individuals are alike, some females may respond to a weak stimulus, whereas others require a more intense exposure. In the spruce budworm (Harvey, 1957), diapause-free development occurs under a long day regime (*i.e.*, greater than 15 hr of light) in a portion of the population. As day length is increased the number of non-diapausing insects increases, reaching 100% in continuous light. Universal diapause occurs when day length is less than 15 hr. Current progress in our laboratory indicates that short photoperiods (less than 12 hr light) are effective in inducing the production of resting eggs by laboratory-reared *L. aestiva*. This will be reported on at length in a subsequent paper.

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SUMMARY

The calanoid copepod, *Labidocera aestiva* was collected from Vineyard Sound, Massachusetts between July and December 1978. Adult size (cephalothorax and total body length) was inversely proportional to surface water temperature at the time of collection. The major switch from subitaneous to resting egg production occurred during late August to early September, but a small percent of subitaneous eggs continued to be produced throughout the fall. Resting eggs were cold-resistant and when chilled at 5° C hatched synchronously when warmed to 21° to 23° C. Individual resting eggs kept continuously at 19° C took longer to hatch and emergence was asynchronous. The resting eggs of *L. aestiva* appear to be in a state of diapause similar to many insects, and it is suggested that photoperiod is the primary cue inducing the production of resting eggs.

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LOCOMOTION IN THE PRIMITIVE PULMONATE SNAIL *MELAMPUS BIDENTATUS*: FOOT STRUCTURE AND FUNCTION

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Most land pulmonates crawl by means of muscular waves that travel along the foot from posterior to anterior. These waves, classified as direct waves by Vlès (1907), have been analyzed by Jones (1973) for the land slug *Agriolimax*. A great variety of wave types has been described for prosobranch gastropods; the most primitive type is thought to be the retrograde type, in which the waves travel in a direction opposite to the gastropod's progress (Miller, 1974; Trueman, 1975). Jones and Trueman (1970) have analyzed the mechanism of retrograde waves in the limpet *Patella* and have reviewed gastropod locomotory waves (Jones, 1975; Trueman, 1975).

The purpose of the work reported here has been to analyze the unusual locomotion of the snail *Melampus bidentatus*. This basommatophoran snail is a member of one of the most primitive families of pulmonates, the Ellobiidae. Information on its mode of locomotion may cast light on the origin of the locomotory behavior exhibited by the more advanced land pulmonates.

Although Morton (1955) indicated that land-inhabiting ellobiids, including *Melampus*, crawl in a way that others have classified as retrograde waves (Jones, 1975), my examination of events in *Melampus* locomotion has not supported that classification. Rather, *Melampus* exhibits a form of locomotion that fits neither the direct nor retrograde wave category nor any of the other locomotor categories of Miller (1974). *Melampus* locomotion consists of a repeated sequence of events, the crawl-step, in which the posterior of the foot slides along but the anterior is lifted and placed. Hydraulic forces, described by Chapman (1958; 1975) for a variety of soft-bodied animals, are an important component in the locomotion of *Melampus*. The blood is acted on by the columellar muscles as well as the intrinsic pedal musculature, and the transverse subdivision of the foot allows the posterior-to-anterior transfer of blood to be coordinated with the muscular events.

MATERIALS AND METHODS

Animal. Adult specimens of *Melampus bidentatus* Say with shell lengths of 7 to 11 mm were collected in salt marshes of Woods Hole, Massachusetts. They were maintained in large, covered fingerbowls lined with filter paper moistened with 75% sea water (Instant Ocean) and provided with napa (Chinese) cabbage and crushed eggshells. A photoperiod of 8L:16D and constant temperature of 16° C suppressed reproductive activity (Apley, 1968).

Photographic Techniques. Crawling snails were filmed with a Bolex Macro-

zoom 160 super 8 mm camera at 18 frames per second. Side and bottom views were obtained simultaneously by the use of mirrors.

Marking the Foot. Snails were anaesthetized according to the method of Price (1977). Three or more spots of India ink were injected just beneath the surface of the sole. The animals were allowed to recover overnight in fresh 75% sea water.

Anatomy. Pedal and columellar muscles were studied in live and narcotized snails and specimens fixed in alcoholic Bouins. Serial sections of snails quick-frozen in liquid nitrogen while crawling (Jones, 1973) provided information on the muscle contraction patterns during locomotion. The 10 μ m sections were stained with Mallory's triple stain (Pantin, 1948).

Force Recordings. Snails were allowed to crawl over a plexiglass platform having a 2-mm gap into which a 1-mm bar was inserted. The bar was attached to a force transducer (Statham Micro-Scale Accessory Model UL5) connected to an amplifier (Gilson IC-MP module). The resulting forces were recorded on a chart recorder (Gilson ICT-SH). The transducer was connected so as to measure either upward and downward forces or backward and forward forces as the snail's foot passed across it. The translucent platform on which the snail crawled was positioned just above the writing surface of the recorder so that the crawling snail was filmed with the chart recorder's activity in the background. The absolute magnitude of the forces was not relevant to this analysis.

RESULTS

Stages in the crawl-step

As illustrated in Figure 1, the foot of *Melampus bidentatus* is divided anatomically into an anterior region (the propodium) and a posterior region (the metapodium) by a permanent transverse groove. The mouth region surrounded by the oral veil or lappets forms an important part of the locomotory surface.

In *Melampus* locomotion a single series of muscular events propagates along the foot at one time. I have called this mode of locomotion a crawl-step because the anterior part of the foot is lifted free of the substratum. Characteristic postures assumed by the foot during the crawl-step have been identified in frame-by-frame analysis of motion picture films of crawling snails. Postures identified with three easily recognized stages of the crawl-step are shown in Figure 1. Each stage is characterized by the following events:

Stage I. Metapodial shortening propodial extension. The posterior third of the metapodium shortens as a single movement and the propodium simultaneously extends to its longest dimension. The head and oral veil are lifted off the substratum.

Stage II. Metapodial extension. A series of local muscular contractions within the metapodium produce one or more waves that ripple forward along the sides of the metapodium from the medial region of the metapodium toward the transverse groove. The region of the metapodium ahead of the elevations bulges outward and when the bulge reaches the transverse groove the posterior edge of the propodium is displaced upward and forward by the expanded metapodium. Mean-

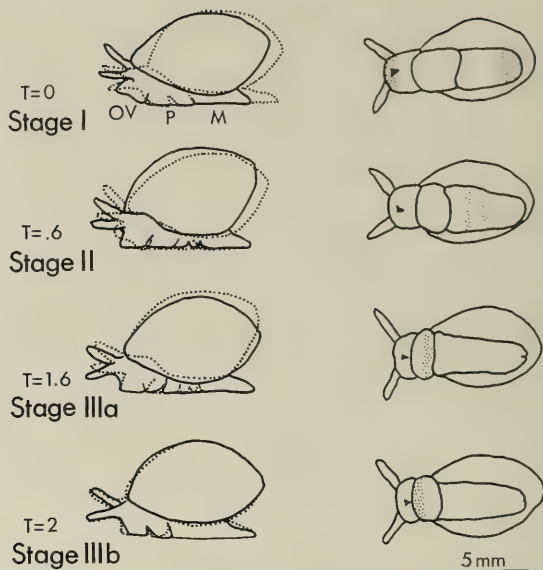


FIGURE 1. The crawl-step of *Melampus bidentatus*. Side and bottom views traced from motion picture frames for Stage I: metapodial shortening/propodial extension, Stage II: metapodial extension, and Stage III: propodial elevation, posterior (a) and then anterior (b) phase. Cumulative elapsed time in seconds is given to the left. Dotted lines (side views) give the snail's posture at onset of the movement characteristic of each stage and the unbroken lines represent the posture upon completion of the movement. Stippled regions (bottom view) indicate portions that were lifted during each stage. Subdivisions of the locomotory surface are labeled in the bottom view of Stage I: OV, oral veil-mouth region; P, propodium of the foot; M, metapodium of the foot.

while the posterior region of the metapodium narrows and flattens dorsoventrally and the shell tilts forward as the oral veil is lowered to the substratum.

Stage III. Propodial elevation. The posterior region of the propodium is elevated first (IIIa, Fig. 1) and then the anterior region is elevated (IIIb, Fig. 1). The oral veil-mouth region forms an area of contact with the substratum throughout this stage. The shell tilts backwards.

In the motion picture from which the tracings shown in Figure 1 were made, the snail progressed 2.2 mm in 2.3 sec. Snails with shell lengths of 10 to 11 mm typically crawled 2 to 4 mm/step at a rate of 10 to 20 steps/min at 20° C.

Foot morphology

Figure 2 shows the relationship of the columellar muscles to the foot. In *Melampus* the columella itself is largely resorbed during development, leaving

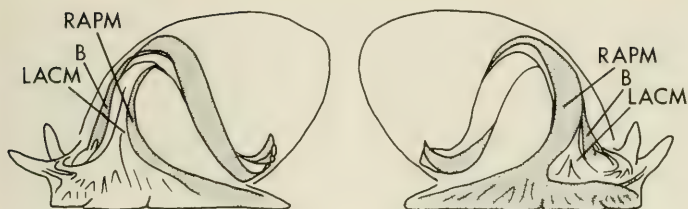


FIGURE 2. Relationship between columellar muscles and the foot in *Melampus*. A, view of animal's left side; B, right side. Left anterior cephalic muscle (LACM) is unshaded, the right anterior and right and left posterior muscle (RAPM) stippled, and buccal muscle (B) crosshatched.

the columellar muscles attached to the inner wall of the shell (Morton, 1955). The muscles follow the inner surface of the shell within the body whorl in approximately a 360° turn from origin to insertion in the extended foot. In addition to the buccal retractor muscle, two major subdivisions of the columellar muscle are apparent in *Melampus*. The left anterior and cephalic muscle (LACM, Fig. 2) originates in several adjacent bundles and further subdivides into muscle bands that insert around the head, into the left tentacle and oral veil, the left side of the propodium and approximately the anterior third of the left side of the metapodium. The right anterior and right and left posterior muscle (RAPM, Fig. 2) has a single origin on the shell and divides into muscle bands that insert on the right tentacle, the right oral veil, the right propodium, the anterior portion of the metapodium and bilaterally in the posterior region of the metapodium. Thus the muscles enter the foot in a quite asymmetrical pattern with those muscles on the left side that support the shell being the more massive. The columellar muscle system of *Melampus* probably includes both the longitudinal and columellar muscle groups described for *Lymnaea* by Plesch, Janse and Boer (1975).

In serial sections of snails quick-frozen in the act of crawling, there is no evidence that intrinsic bands of muscle fibers form discrete layers above the sole of the foot. Rather, the fibers that could be traced appear to be derived from columellar muscles. This is apparent in the parasagittal section which cuts through the left anterior and cephalic columellar muscle (Fig. 3C). The muscle fibers form a meshwork enclosing small, spherical blood spaces, shown in a region of the metapodium (Fig. 3E). These are similar to those in the foot of the limpet, *Patella* (Jones and Trueman, 1970). These small spaces are contrasted with the large blood sinuses that are present in the anterior region of the foot (Fig. 3A, D).

When sections of snails frozen in different stages of the step cycle are compared, one of the most obvious differences is in the orientation of the transverse groove. In Stage I, the metapodial shortening/propodial extension results in a propodium that is long and a transverse groove that is shallow and anteriorly-slanting (Fig. 3A). In Stage IIIa elevation of the transverse groove and posterior region of the propodium pulls the groove into a deep backward-slanting indentation (Fig. 3C). As the propodial elevation moves into the anterior propodium (Stage

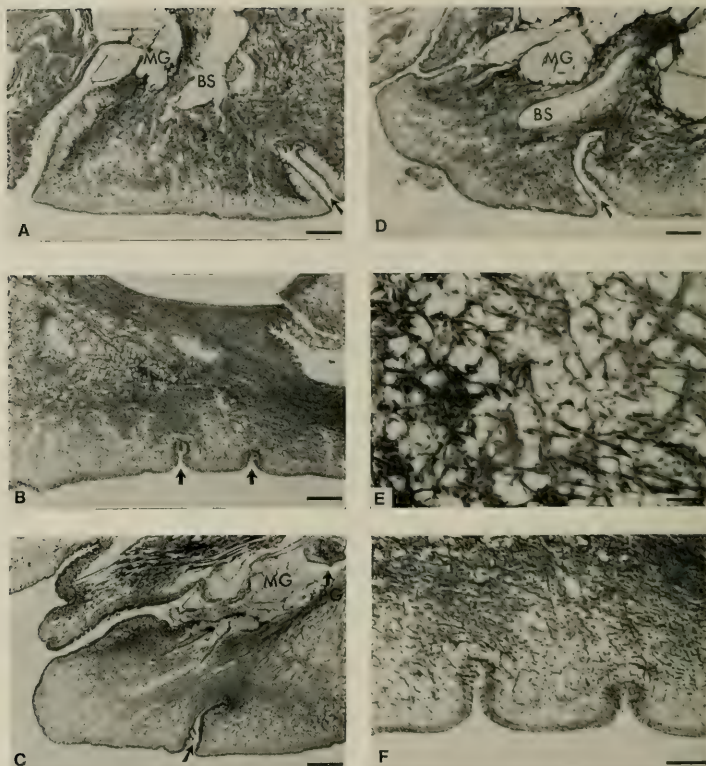


FIGURE 3. Sections prepared from specimens frozen in liquid nitrogen while crawling. In each case, anterior is to the left. A. Stage I posture, mid-sagittal section showing propodial region of foot and transverse groove (arrow). Scale bar 250 μ . B. Stage II posture, mid-sagittal section showing metapodial region with two metapodial waves (arrows). Scale bar 250 μ . C. Stage IIIa posture, left para-sagittal section through left anterior and cephalic muscle bands in the propodial region of the foot. Arrow indicates transverse groove. Scale bar 250 μ . D. Stage IIIb posture, mid-sagittal section showing propodial region of the foot and transverse groove (arrow). Scale bar 250 μ . E. Detail of metapodial tissue showing small blood spaces. Scale bar 25 μ . F. Detail of muscle contraction pattern in the metapodial waves in Figure 3B. Scale bar 75 μ . Abbreviations used: BS, large blood sinus; MG, suprapedal mucus gland; PG, pedal ganglion.

IIIb) the transverse groove region is relaxed and assumes a condition intermediate between that of Stage IIIa and Stage I (Fig. 3D).

Metapodial contractile waves are shown in Figure 3B and the muscle pattern

contributing to them is apparent at higher magnification in Figure 3F. The sole of the foot is lifted straight up rather than pulled in a slanting angle forward. It appears that the elevations are caused by a local tightening of the mesh-work of fibers within the foot. Anterior to the elevations, the blood spaces are larger than in the contracted regions behind the waves. A consequence of anterior movement of such a pattern of waves would be the forward displacement of blood.

Marked-foot experiments

In this and the following sections an attempt was made to characterize more precisely the sequence of events and the forces underlying forward progression during the crawl-step. The aim of marked-foot experiments was to analyze temporally the changes in the relative size of the propodium and metapodium and the movement of points within those regions. This was achieved by filming the foot of snails that had spots of ink injected into the sole. In every fourth frame of the film the position of the ink spots was determined, relative to a fixed point behind the snail's foot. Sample data from five crawl-steps are illustrated in Figure 4. The beginning and end of one crawl-step is included between the vertical dashed lines.

The following features are apparent from such an analysis: First, shortening of the posterior metapodium, measured as advancement of the end of the foot, is mirrored in time and magnitude by advancement of the anterior edge of the propodium. Thus during posterior shortening the length of the snail's foot does not change. The remaining events in the crawl-step are all forward shifts of intermediate points along the foot, relative to the fixed posterior and anterior

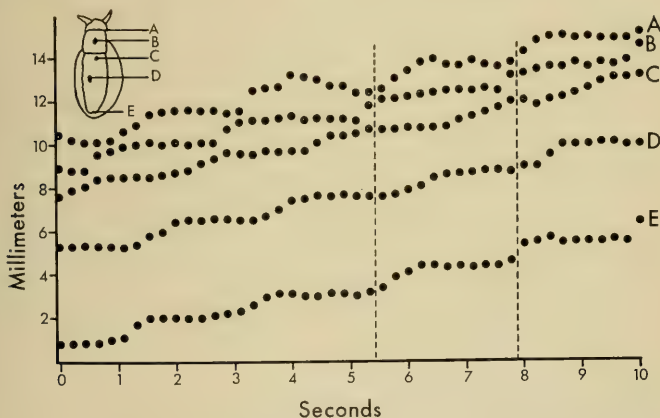


FIGURE 4. Progress of the anterior (A) and posterior (E) edges of the foot and three spots of injected ink (B-D) was charted for every fourth frame of an 18 frames/sec motion picture film. The events of one complete crawl step occur between the vertical dashed lines.

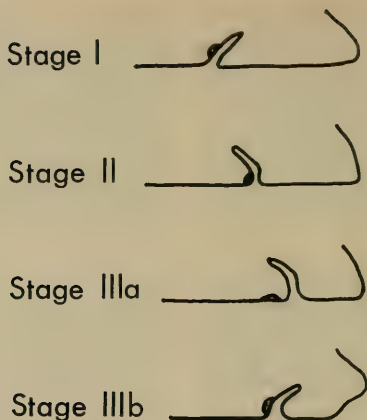


FIGURE 5. Diagrammatic illustration of the relationship of an ink spot to the transverse groove. Motion picture films indicated that during locomotion the spot of ink disappeared within the transverse groove from Stage IIIb through Stage I and was visible during Stages II and IIIa.

edges of the foot. These forward shifts proceed from posterior to anterior: the metapodial waves carry point D and then point C forward. Finally, point B moves forward as the anterior propodium is lifted off the substratum.

Films of a snail with a differently placed ink spot shed light on events occurring in the region of the transverse groove during locomotion. The ink spot was in the anterior metapodium almost within the transverse groove. During locomotion, the spot appeared and disappeared with each crawl-step. The position of the spot during the crawl-step is illustrated diagrammatically in Figure 5. It became visible during metapodial extension in Stage II and was eclipsed by expansion of the posterior propodium as the anterior propodium was elevated (Stage IIIb). Both this and the histological evidence indicate that although the transverse groove is a constant feature of the foot, a greater or lesser region of the pedal sole anterior or posterior to this region can be drawn up into the groove. Both changes in blood volume in the propodium and metapodium adjacent to the groove, and contraction of columellar muscles which insert close to the groove, alter its configuration.

Upward and downward forces

Direct observations and motion picture films indicate that portions of the *Melampus* foot are elevated during locomotion. Forward sliding movements along the mucus-covered substratum might also be expected to exhibit an upward component. Transducer recordings of upward and downward forces were obtained

to determine which portions of the sole are experiencing upward or downward (weight-bearing) forces during each stage in the crawl-step cycle.

The forces exerted during locomotion were measured as snails crawled across a moveable bar positioned in a slit in a plexiglass platform. Five to six step cycles were required for snails to crawl across the bar and therefore at each successive step the transducer measured forces from a more posterior region of the foot. The pattern of upward and downward deflections recorded in this manner was fairly constant from crossing to crossing and animal to animal. Figure 6A is typical of the recordings that were produced. These data were interpreted as described in the methods. Postures characteristic of Stages I-III for each step were identified with particular points on the force recording as illustrated in Figure 6A.

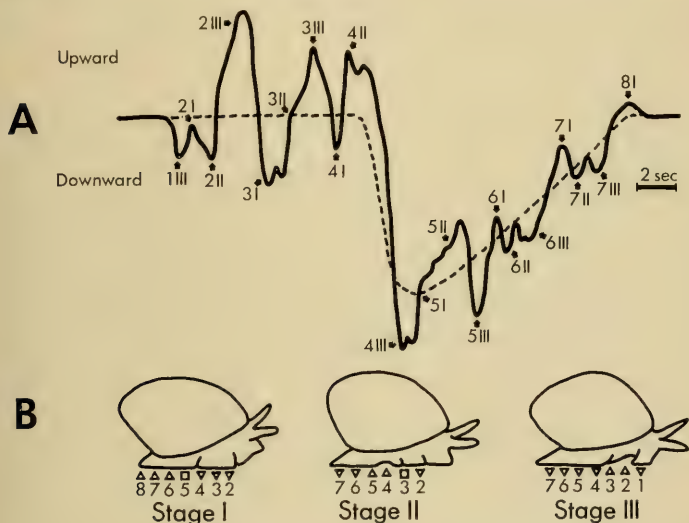


FIGURE 6. A is the force transducer recording of upward and downward forces exerted on a bar as a snail crawled across it. A portion of the snail's foot was in contact with the bar for six complete steps and the end and beginning of two other steps, for a total of 24 sec. Each point at which the simultaneously-recorded motion picture film indicated that the snail assumed one of the postures characteristic of Stage I, II or III of the crawl step cycle was marked on the transducer recording. The dotted line represents a baseline adjustment that was inserted to compensate for the overwhelming effect of passage of the shell mass onto the bar. In B the information obtained from the force recording is represented as a pattern of upward and downward-pointing triangles showing the distribution of vertical forces exerted by the foot on the substratum at Stages I, II and III of the step cycle. Regions exerting no vertical force are indicated by squares. The position of the triangles or squares under the foot marks the position of the recording bar each time the posture characteristics of a particular stage was assumed.

Note that in the force recording in Figure 6A there is a dramatic downward registration following stage II of the fourth step. At that time the shell weight shifts forward onto the bar. From then until the foot is pulled off the bar, all the upward deflections fail to rise above the original baseline. The dotted line in Figure 6A was inserted as a relative baseline in an attempt to correct for the overwhelming effect of shell weight on the force recordings. Using that correction, the results of these experiments were summarized in Figure 6B. The following conclusions can be drawn: In metapodial shortening/propodial extension the propodium is weight-bearing while the posterior region of the metapodium exerts an upward force on the substratum. In metapodial lengthening the anterior region of the propodium and the posterior region of the metapodium are weight-bearing while the waves moving forward in the anterior region of the metapodium exert an upward force. In propodial elevation the oral veil and the entire metapodium are weight-bearing during elevation of the posterior and anterior propodium.

Forward and backward forces

Lissmann (1946) defined the sliding progression of a snail's foot along a substratum as being the product of forces acting parallel to the ground, including internal and external forces acting to change the shape of the snail's body and the reactions from the ground. The concept of the internal forces has been advanced and direct measurements of fluid pressure made for a variety of animals since that time (see Chapman, 1975). The reactions of the ground to the passage of the snail's foot were measured in this study to indicate what was happening along the length of the foot at each stage in the crawl-step cycle. Lissmann's (1946) reactions from the ground were defined as the static reaction, which is the force exerted on the substratum in a backward direction by stationary areas while other regions of the foot are advanced, and sliding friction, which is the forward force that moving portions of the foot exert. These backward and forward forces were recorded and interpreted in a manner similar to that described for upward and downward forces, but with the transducer measuring forces in the horizontal plane.

In general the sole of the foot did not noticeably protrude into the space in the plexiglass slit adjacent to the recording bar, but rather moved smoothly onto the bar. There were two notable exceptions to this generalization: if the spacing of the animal's approach to the bar was such that anterior metapodial advancement or propodial advancement began at the edge of the slit, then the foot region bulged downward into the gap and thereby displaced the bar forward by pushing it from behind. A sample recording of the forward and backward forces is shown in Figure 7A. The labels on the recording show the points at which the snail's posture matched the characteristic postures for stages I, II, and III in each of the six crawl steps. The distribution of forces beneath the foot at each stage is illustrated in Figure 7B. During metapodial shortening/propodial extension, the posterior metapodium and the anterior propodium register forward sliding friction while the anterior metapodium and posterior propodium show the static reaction. In metapodial extension the entire metapodium registers a forward force and the propodium registers a backward force. In propodial elevation the propodium is the only region showing forward sliding friction.

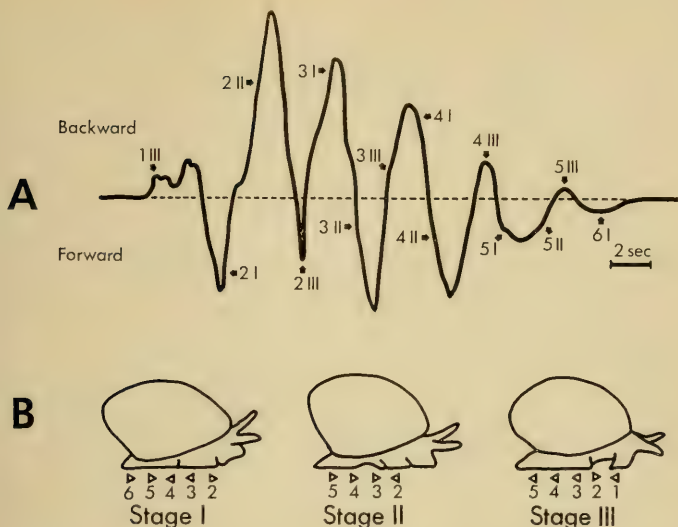


FIGURE 7. A is the force transducer recording of forward and backward forces exerted by a snail's foot as it crawled across a bar. The snail required four complete crawl-step cycles and portions of two other cycles to complete the crossing. Information was obtained in a manner similar to that described for Figure 6. B is the pattern of forward and backward forces exerted by the foot on the substratum in Stages I, II and III of the crawl-step.

DISCUSSION

The crawl-step of *Melampus* is a complex sequence of events compared with the direct pedal waves of most land pulmonates. Figure 8 gives a model for *Melampus* locomotion that was constructed from the histology, cinematography and force transducer recordings presented in the results.

According to this model the Stage I metapodial shortening/propodial extension result from a single muscular event: contraction of columellar muscles in the posterior half of the metapodium. This action not only draws the end of the foot upward and forward, as indicated by the transducer recordings, but it causes some blood to leave the posterior region of the foot. This blood could flow across the low transverse groove into the propodium and into the anterior region of the body. The pattern of forces recorded from the foot at this stage indicates that the weight is primarily borne by the posterior propodium and that it and the anterior metapodium experience a backward drag (the static reaction). The combination of downward and forward forces recorded from the anterior propodium are what would be expected in a region experiencing invasion of blood from the posterior. The observation that the propodium bulged downward into the crack

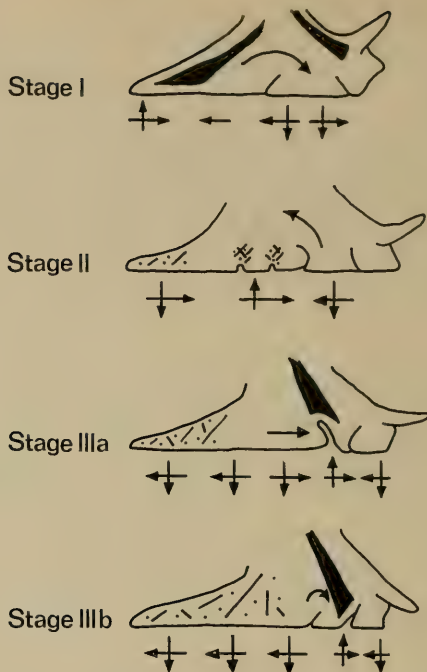


FIGURE 8. Model of mechanical events producing locomotion in *Melampus*. Arrows below the foot show forces exerted on the substratum; arrows within the body show fluid dynamics; dark regions in the body indicate muscle contraction patterns. See text for discussion.

adjacent to the recording bar indicates that relaxed tonus in the propodium may contribute to the hydraulic expansion.

In Stage II, metapodial extension is produced by low-amplitude waves travelling from the middle toward the anterior metapodium. Their action is depicted as squeezing blood forward within the ventral portion of the foot until it accumulates behind the transverse groove. The resulting change in orientation of the groove causes blood in the posterior propodium to move up into the large blood sinuses in the dorsal propodium and anterior metapodium. The snail's weight rests on the anterior propodium and the posterior metapodium while the middle of the foot shifts forward. The observed narrowing of the metapodium that begins in this stage and continues through Stage III probably exerts a tonic force on the blood and favors forward movement of the foot. This tonic force may be produced

by body wall musculature rather than by pedal muscle fibers derived from the columellar muscles.

In Stage III, propodial elevation results from contraction of columellar muscle bands, chiefly those of the left anterior and cephalic columellar muscle. The transverse groove region is elevated in Stage IIIa. This allows the anterior edge of the metapodium to expand into the space formerly occupied by the posterior propodium. Again, the combination of downward and forward forces indicates that metapodial extension results from an hydraulic event. By Stage IIIb the more anterior region of the propodium is elevated by columellar muscles and the posterior region returns to the substratum in advance of its former position. This forward displacement is due, at least in part, to the extension of the metapodium in Stage IIIa. Throughout Stage III the oral veil and the posterior metapodium are static, weight-bearing regions that support the forward progression of the anterior region of the foot.

The hydraulic expansion of the anterior region of the foot of *Melampus* is similar to hydraulic events described for burrowing in the naticid snail *Polinices josephinus* (Trueman, 1968). The work of Schiemenz (1884) and, more recently, Russell-Hunter and Russell-Hunter (1968) and Russell-Hunter and Apley (1968) revealed that in naticids water is taken up into special channels to aid in the expansion of the foot. Both the burrowing *Polinices* and the crawling *Melampus* advance in a similar stepwise fashion.

The deeply cleft transverse groove and elevation of the propodium in a stepping locomotion are features that have arisen independently in several lines of Ellobiidae that are adapted to a hard substratum (Morton, 1955). Morton (1955) suggests that it may be especially adaptive in progression over broken or irregular surfaces. The model presented above indicates that the transverse groove has a valve-like function, rendering the metapodium relatively independent of the propodium during metapodial extension. This enhanced control over hydraulic events may have been important in the transition from the marine habitat to land in the Ellobiidae.

Morton (1955) describes the locomotion of ellobiids with a divided foot as "... fixing down the anterior third of the foot well in advance of the animal and drawing the remainder forwards upon it" (p. 151). This brief description neglects the hydraulic expansion of the propodium and thereby exaggerates the superficial similarity between *Melampus* locomotion and the "loping" locomotion of another primitive pulmonate, *Otina otis*. According to Vlès' (1913) account of *Otina* locomotion, the transversely subdivided foot is used in a stepping fashion in which the anterior is lifted and placed forward, rendering the foot long, and then the posterior is pulled forward, rendering the foot short. The hydraulic component that is responsible for maintaining the *Melampus* foot at a relatively constant length is apparently absent in *Otina* locomotion. Furthermore, no matter in what order the events of *Melampus* locomotion are considered, the contraction pattern within the propodium clearly passes from posterior to anterior and therefore the crawl-step locomotion exhibited by this snail cannot be considered a retrograde wave. Thus the locomotion of *Melampus* cannot be cited to support the supposition that the retrograde wave locomotion seen in stylommatophoran gastropods during escape behavior is a primitive type of locomotor behavior within the pulmonates.

The only feature of *Melampus* locomotion that is similar to the locomotion of

higher land snails and slugs is the anteriorly-travelling ripple that produces metapodial lengthening. This wave of contraction is apparently produced by intrinsic pedal musculature and, although the *Melampus* foot appears to lack the specialized musculature that produces the waves in *Agriolimax* (Jones 1973), observation of the side view clearly shows that the elevated region of the wave is compressed and its forward movement forces a bolus of fluid forward. The fact that these metapodial waves do not originate at the end of the foot is very significant in an attempt to relate them to the direct waves of stylommatophorans. This is the case because, as Jones (1975) has pointed out, the multiple direct wave pattern of higher pulmonates could not be derived by adding waves to the single direct wave type of locomotion such as that exhibited by *Onchidella*, in which the wave begins by shortening of the posterior region of the foot (Vlés, 1907). Such an action renders the foot shorter during locomotion than when it is at rest and the propagation of many waves at the posterior of the foot before the first wave passes off the anterior would cause the foot to become prohibitively short. The wave pattern in higher pulmonates, in contrast, is initiated at the onset of locomotion at a site near the anterior part of the foot, where constriction of the elevated region results in a compensatory stretching of a more posterior part of the foot and the foot length remains constant (Lissmann, 1945; Jones, 1975). Thus the metapodial waves of *Melampus* could represent the survival of the motor pattern that gave rise to multiple direct wave locomotion in stylommatophorans. Morton's (1955) description of locomotion in ellobiids that lack a transverse groove suggests that this wave pattern may be more pronounced in less specialized members of the family.

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SUMMARY

The foot of *Melampus* is subdivided into an anterior propodium and a posterior metapodium by a permanent transverse groove. Locomotion in *Melampus* consists of repetition of a cycle of events that pass from posterior to anterior; this cycle has been named a crawl-step. Three stages in the crawl-step have been identified: Metapodial shortening is produced by the action of columellar muscles and this action forces blood anteriorly to extend the propodium. Metapodial lengthening is produced by muscle action within the metapodium and extends the metapodial region forward at the expense of the propodium. Propodial elevation is produced by columellar muscles and prepares the propodium to "step" forward while fluid invasion occurs in the first stage.

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THE ECTOPARASITISM OF *BOONEA* AND *FARGOA* (GASTROPODA: PYRAMIDELLIDAE)¹

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Pyramidellids are now well known to be ectoparasites feeding on the body fluids of invertebrates (Ankel, 1938, 1949a, 1949b, 1959; Fretter and Graham, 1949, 1962; Fretter, 1951). Most of the verified hosts are polychaetes, gastropods and bivalves, but there also appear to be various minor host groups such as polycladophorans and echinoderms (Robertson and Orr, 1961). A defect of many of the reports of hosts and even of "feeding" experiments is that no definite observations on feeding were made. It is unsatisfactory to report a "host" when a pyramidellid is merely found "on" or "with" an invertebrate, whether it be in the field or in the laboratory. Pyramidellids, being foraging animals, sometimes assume positions on living substrates other than their hosts. Also, there can be behavioral preludes to feeding that do not culminate in feeding. A dilemma is that feeding cannot be verified *in situ* in the field, while in the laboratory some pyramidellids will feed on "hosts" they would never naturally encounter; the feeding of other pyramidellids is observed with great difficulty in the laboratory, even when the probable natural host is offered. Under the circumstances, what should be done is to observe for consistent associations with "hosts" in the field and then to determine whether these "hosts" are fed on in the laboratory.

The degree to which pyramidellids are host-specific remains unresolved. Fretter and Graham (1949, 1962), even while recording the European species *Odostomia ambigua* (*O. "culinoides"*) on *Pecten maximus*, *Chlamys opercularis* and *Ostrea edulis*, emphasized that pyramidellids are host-specific. Berry (1955) went so far as to suggest that host-specificity accounts for the large number of pyramidellid species. There may be pyramidellids that are specific to one host species, but there are a few suggestive data that others are not. Ankel and Christensen (1963) observed *Odostomia rissoides* (*O. "scalaris"*) in Denmark feeding on five species in the laboratory: *Lacuna vincta* (*L. "divaricata"*), *Littorina saxatilis*, *Hydrobia ulvae*, *Rissoa membranacea* and *Chlamys opercularis*. All but the last species live in the same habitat. There are, too, literature records of *O. rissoides* with *Mytilus edulis*. Ankel and Christensen (1963) also assembled literature data showing that *O. ambigua* had been found with four pectinid species, *Mytilus edulis*, *Ostrea edulis*, *Hiatella ("Saxicava") rugosa* and *Turritella communis*. Minichev (1971) found *Odostomia fujitani* on various mollusks in the Sea of Japan, and under experimental conditions it fed readily on *Littorina brevicula*, *Tegula rustica*, *Umbonium costatum*, *Turritella fortillirata*, *Arca boucardi*, etc. According to LaFollette (1979), the southern California species *Chrysallida cincta* has at least six hosts: *Haliotis corrugata*, *H. fulgens*, *Tegula eiseni*, *Norrisia norrisi*, *Astraea undosa* and *A. gibberosa* (all archaeogastropods).

¹ Woods Hole Oceanographic Institution Contribution No. 4313.

The purposes of the present paper are: to tabulate literature records of the "hosts" of all eastern North American odostomioids (*Boonea* and *Fargoa* species), to present (with emphasis on *Boonea seminuda* and *B. bisuturalis*) original field data on their occurrences and frequencies with "hosts", to present laboratory data on the "hosts" that were fed upon, and to present laboratory data on the preferences that *B. seminuda* and *B. bisuturalis* had for two hosts. Of prime interest was the sympatric occurrence and abundance of these two species, and how they divide resources. Two species in the related genus *Fargoa* were also of interest by way of comparison, but fewer data are available about them.

Justification for separating the genera *Boonea* and *Fargoa* from *Odostomia* has recently been presented elsewhere (Robertson, 1978). The systematics of the species reported on here was also treated in the same paper. *Boonea impressa* is considered one species, although the populations in North Carolina and northwest Florida have planktotrophic larval development while a Texas population is lecithotrophic. The nomenclature and systematic sequences of the non-pyramidellid mollusks in the tables follow Abbott (1974) except that *Bittium* is used instead of *Diastoma* (Houbrick, 1977).

MATERIALS AND METHODS

Odostomioids were collected in the vicinities of Plymouth and Woods Hole, Massachusetts, and Beaufort and Wilmington, North Carolina. Correspondents sent living specimens and habitat data from Sapelo Island, Georgia, and Galveston, Texas. In all, seven species were collected alive, and careful note was taken on whether they were consistently on a particular living substrate—the presumptive host. "Hosts" were obtained near Woods Hole, on the New Jersey coast, near Beaufort, and from Texas.

Quadrats were used to determine the abundance of *Boonea* relative to its hosts and to other mollusks in the same habitats. The quadrats were placed at random in habitats suitable for two *Boonea* species (for *B. seminuda*, the muddy sand shallow subtidal zone where *Crepidula fornicata* is common at the N end of Quissett Harbor, 3 km NE of Woods Hole, mid-August 1978; for *B. bisuturalis*, the stony low intertidal zone, underlain by sand and peat, where *Littorina littorea* is common at the shore opposite Flume Pond, S of Gunning Point, 4 km NNE of Woods Hole, early and late August, 1978). There was time only for two quadrats for each species. Three of the quadrats were 1.0 × 1.0 m; the fourth, because of a rising tide, had to be 0.5 × 0.5 m. All mollusks (except *Bittium alternatum* in the *Boonea seminuda* habitat) in each quadrat were collected, identified, counted and weighed (the weights include the shells). The five species of *Boonea* and *Fargoa* whose presumptive hosts were determined in the field were kept with these hosts in bowls of sea water in the laboratory. After it was learned that *Boonea* would feed on mollusks other than the presumptive hosts, all mollusks found in *Boonea* habitats plus a selection of species from other habitats were offered. Two polychaete species found in the same habitat as *B. bisuturalis* were also offered, as was the ascidian *Molgula*. The two *Fargoa* species were offered the serpulid *Hydroides dianthus*, a few other (unidentified) polychaetes, and various mollusks (*Littorina littorea*, *Crepidula fornicata* and *Anachis avara*).

TABLE I
Literature records of eastern North American species of Boonea and Fargoa with "hosts" (nomenclature brought up to date).

Species	"Host"	Area	Association observed in field (F) or lab. (L).	True feeding observed	Author
<i>B. seminuda</i>	<i>Argopecten irradians</i>	Beaufort, NC	F	no	Hackney, 1944, p. 60
<i>B. seminuda</i>	<i>Crepidula fornicata</i>	Woods Hole, MA	F, L	yes	Robertson, 1957
<i>B. seminuda</i>	<i>Argopecten gibbus</i>	off Ocracoke and Core Banks, NC	F, L	yes	Wells and Wells, 1961; Wells, Wells and Gray, 1964, p. 577
<i>B. seminuda</i>	<i>Crepidula fornicata</i>	West Dennis, MA	F, L	yes	Merrill and Boss, 1964; Boss and Merrill, 1965
<i>B. seminuda</i>	<i>Crucibulum striatum</i>	Cape Cod, MA	L	yes	Merrill and Boss, 1964; Boss and Merrill, 1965
<i>B. seminuda</i>	<i>Crassostrea virginica</i>	West Dennis, MA	L	no	Boss and Merrill, 1965
<i>B. seminuda</i>	<i>Mercenaria mercenaria</i>	Cape Cod, MA	L	no	Boss and Merrill, 1965
<i>B. seminuda</i>	<i>Argopecten irradians</i>	Cape Cod, MA	L	no	Boss and Merrill, 1965
<i>B. seminuda</i>	<i>Modiolus modiolus</i>	Cape Cod, MA	L	no	Boss and Merrill, 1965
<i>B. seminuda</i>	<i>Placopecten magellanicus</i>	Cape Cod, MA	L	no	Boss and Merrill, 1965
<i>B. seminuda</i>	<i>Argopecten irradians</i>	North Carolina	F	no	Porter, 1974, 1976
<i>B. bisuturalis</i>	<i>Geukensia demissa</i>	Long Island, NY	F	no	Pelcener, 1928, p. 172
<i>B. bisuturalis</i>	<i>Mytilus edulis</i>	Long Island, NY	F	no	Pelcener, 1928, p. 172
<i>B. bisuturalis</i>	<i>Mya arenaria</i>	Gulf of St. Lawrence	F	no	Medcof, 1948
<i>B. bisuturalis</i>	<i>Crassostrea virginica</i>	New England	F, L	yes	Loosanoff, 1956
<i>B. bisuturalis</i>	<i>Crassostrea virginica</i>	West Dennis, MA	F, L	yes	Merrill and Boss, 1964; Boss and Merrill, 1965
<i>B. bisuturalis</i>	<i>Littorina littorea</i>	Woods Hole, MA	F, L	yes	Scheltzema, 1965
<i>B. bisuturalis</i>	<i>Bittium alternatum</i>	Woods Hole, MA	L	yes	Scheltzema, 1965
<i>B. bisuturalis</i>	<i>Crepidula fornicata</i>	West Dennis, MA	L	no	Boss and Merrill, 1965
<i>B. bisuturalis</i>	<i>Mercenaria mercenaria</i>	Cape Cod, MA	L	no	Boss and Merrill, 1965
<i>B. bisuturalis</i>	<i>Argopecten irradians</i>	Cape Cod, MA	L	no	Boss and Merrill, 1965
<i>B. bisuturalis</i>	<i>Crucibulum striatum</i>	Cape Cod, MA	L	no	Boss and Merrill, 1965
<i>B. bisuturalis</i>	<i>Modiolus modiolus</i>	Cape Cod, MA	L	no	Boss and Merrill, 1965
<i>B. bisuturalis</i>	<i>Crepidula plana</i>	Cape Cod, MA	L	no	Boss and Merrill, 1965

TABLE I—Continued

Species	"Host"	Area	Association observed in field (F) or lab. (L)	True feeding observed	Author
<i>B. bisuturalis</i>	<i>Placopecten magellanicus</i>	Cape Cod, MA	L	no	Boss and Merrill, 1965
<i>B. bisuturalis</i>	<i>Littorina littorea</i>	Woods Hole, MA	F, L	yes	Robertson, 1967
<i>B. bisuturalis</i>	<i>Ilyanassa obsoleta</i>	Duxbury Beach, MA	F	no	Bullock and Boss, 1971
<i>B. bisuturalis</i>	<i>Mytilus edulis</i>	Duxbury Beach, MA	F	yes	Bullock and Boss, 1971
<i>B. bisuturalis</i>	<i>Littorina littorea</i>	Duxbury Beach, MA	F	no	Bullock and Boss, 1971
<i>B. bisuturalis</i>	<i>Urosalpinx cinerea</i>	Duxbury Beach, MA	F	no	Bullock and Boss, 1971
<i>B. bisuturalis</i>	<i>Crepidula conchata</i>	Duxbury Beach, MA	F	no	Bullock and Boss, 1971
<i>B. bisuturalis</i>	<i>Crepidula fornicata</i>	Duxbury Beach, MA	F	no	Bullock and Boss, 1971
<i>B. bisuturalis</i>	<i>Nassarius trivittatus</i>	Duxbury Beach, MA	F	no	Bullock and Boss, 1971
<i>B. bisuturalis</i>	<i>Ilyanassa obsoleta</i>	[Woods Hole, MA]	L	yes	Abbott, 1972, p. 45
<i>B. impressa</i>	<i>Crassostrea virginica</i>	Florida	F	no	Dall, 1883, p. 331
<i>B. impressa</i>	<i>Crassostrea virginica</i>	South Carolina	F, L	yes	Hopkins, 1956
<i>B. impressa</i>	<i>Crassostrea virginica</i>	Chesapeake Bay	L	yes	Allen, 1958
<i>B. impressa</i>	<i>Bittium varium</i>	Chesapeake Bay	L	yes	Allen, 1958
<i>B. impressa</i>	<i>Crepidula conchata</i>	Chesapeake Bay	L	yes	Allen, 1958
<i>B. impressa</i>	<i>Triphora nigrocincta</i>	Chesapeake Bay	L	yes	Allen, 1958
<i>B. impressa</i>	<i>Urosalpinx cinerea</i>	Chesapeake Bay	L	no?	Allen, 1958
<i>B. impressa</i>	"polychaete" worm	Chesapeake Bay	L	no?	Allen, 1958
<i>B. impressa</i>	<i>Molgula</i>	Chesapeake Bay	L	yes	Allen, 1958
<i>B. impressa</i>	cf. <i>Fargoa gibbosa</i>	Chesapeake Bay	L	yes	Wells, 1959
<i>B. impressa</i>	<i>Crassostrea virginica</i>	Beaufort, NC	F	no	Moore, 1961, p. 33
<i>B. impressa</i>	<i>Crassostrea virginica</i>	Mississippi Sound	F	no	Porter, 1974, 1976
<i>B. impressa</i>	<i>Crassostrea virginica</i>	North Carolina	F	no	Wells and Wells, 1961
<i>B. impressa</i>	<i>Hydroides dianthus</i>	Beaufort, NC	L	yes	Roberge, 1968
<i>F. dianthophila</i>	<i>Hydroides dianthus</i>	Buzzard's Bay, NC	L	no	Wells and Wells, 1969
<i>F. dianthophila</i>	<i>Hydroides microlis</i>	off Core Bank, NC	L	no	Wells and Wells, 1969
<i>F. dianthophila</i>	<i>Hydroides floridanus</i>	N. E. & N. W. Florida	L	no	Wells and Wells, 1969
<i>F. dianthophila</i>	<i>Hydroides crucigera</i>	Wakulla and Franklin Co, FL	L	no	Wells and Wells, 1969
<i>F. dianthophila</i>	<i>Hydroides dianthus</i>	N. W. Florida	L	no	Wells and Wells, 1969
<i>F. bartshi</i>	<i>Hydroides dianthus</i>	[Woods Hole, MA]	[F, L]	[yes]	Robertson, 1978

To be considered in a *feeding position* odostomioids had to be stationary on a "host" for about two or more minutes, and less than a proboscis length away from an accessible portion of the potential "host's" soft tissues. Usually after assuming a feeding position, odostomioids *probing* their "hosts" partially everted and inverted their proboscises one or more times, near or onto the "host's" soft tissues (probing rarely occurs away from any host). *Feeding* was recorded only when the proboscis was fully everted, when the "host" was pierced, and when the buccal pump within the proboscis "vibrated" to and fro with peristaltic waves.

The *Boonea* and *Fargoa* animals and the "hosts" were kept together until feeding, death, or termination of the experiment occurred. The interactions were monitored with a binocular dissecting microscope. Differences in the number of "hosts" available and the time available for observations account for the differences in the "hosts" that were offered each species in Table V.

The movements of two *Boonea* species towards two hosts at a distance were quantified. Round, plastic washbasins (diameter 30 cm) were filled with sea water to a depth of 5 cm. Four *Crepidula fornicata* chains were placed at equal distances around the perimeter and four groups of four large specimens of *Littorina littorea* were placed between them. Sixty individuals of *Boonea seminuda* or *B. bisuturalis* (all about the same size) were placed in the center of each basin and allowed to move freely. The littorinas also moved freely and occasionally one had to be placed back in the water after it crawled up the side of the basin. Otherwise the basins were undisturbed for 3 hrs, after which time the littorinas and crepidulas were removed and the number of *Boonea* individuals on each was counted. All *Boonea* animals were then removed from their hosts and the basin. The basin was washed and new sea water was added. Each experiment was done twice each day. *Boonea* was not allowed to feed except when in the basin (6 hrs per day). There were ten trials for *B. seminuda*, and eight trials for *B. bisuturalis*. The data for each species were tested for homogeneity to determine whether the two sets of data could be lumped and treated as two experiments (Sokal and Rohlf, 1969). Chi-squares were then computed.

RESULTS

There are literature records of three species of *Boonea* with 22 species of mollusks, one species of polychaete and one species of ascidian (Table I). Only 18 of the 44 records were based on actual feeding observations. Table I also shows that two species of *Fargoa* are recorded with four species of *Hydroides*. Only two of the seven records were based on feeding observations.

In the present study, three species of *Boonea* were found alive and with hosts (all of them molluscan; Table II). Four species of *Fargoa* were found alive, but only two of them were with hosts (both *Hydroides dianthus*; Table II).

Four *Boonea seminuda* animals were found in the winter with *Littorina littorea* in the habitat occupied at other seasons by *B. bisuturalis* (shore opposite Flume Pond, near Woods Hole). *B. bisuturalis* was occasionally found in the summer with *B. seminuda* on *Crepidula fornicata* (N end of Quissett Harbor, near Woods Hole).

The quadrat data (Tables III and IV show that *Boonea seminuda* and *B.*

TABLE II

Field observations on *Boonea* and *Fargoa* species found alive in the present study: localities and presumptive hosts.

Species	Area	In feeding position on	Association
<i>B. seminuda</i>	Woods Hole	<i>Crepidula fornicata</i>	common
<i>B. seminuda</i>	Woods Hole	<i>Littorina littorea</i>	rare
<i>B. seminuda</i>	Woods Hole	<i>Argopecten irradians</i>	rare
<i>B. seminuda</i>	Beaufort	<i>Crepidula fornicata</i>	common
<i>B. seminuda</i>	Beaufort	<i>Argopecten irradians</i>	common
<i>B. bisuturalis</i>	Plymouth	<i>Ilyanassa obsoleta</i>	common
<i>B. bisuturalis</i>	Woods Hole	<i>Littorina littorea</i>	common
<i>B. bisuturalis</i>	Woods Hole	<i>Crepidula fornicata</i>	fairly rare
<i>B. bisuturalis</i>	Woods Hole	<i>Argopecten irradians</i>	fairly rare
<i>B. impressa</i> *	Beaufort	<i>Crassostrea virginica</i>	common
<i>B. impressa</i> *	Wilmington	<i>Crassostrea virginica</i>	common
<i>B. impressa</i> *	Sapelo I.	<i>Crassostrea virginica</i>	common
<i>B. "impressa"</i> †	Galveston	<i>Crassostrea virginica</i>	common
<i>B. "impressa"</i> †	Galveston	<i>Ischadium recurvum</i>	rare
<i>B. "impressa"</i> †	Galveston	<i>Geukensia demissa</i>	rare
<i>F. dianthophila</i>	Woods Hole	<i>Hydroides dianthus</i>	common
<i>F. dianthophila</i>	Beaufort	<i>Hydroides dianthus</i>	common
<i>F. bushiana</i>	Beaufort	not found	
<i>F. bartschi</i>	Woods Hole	<i>Hydroides dianthus</i>	common
<i>F. bartschi</i>	Beaufort	not found	
<i>F. bartschi</i>	Wilmington	<i>Hydroides dianthus</i>	common
<i>F. bartschi</i>	Texas	not found	
<i>F. gibbosa</i>	Beaufort	not found†	

* Planktotrophic.

† Lecithotrophic.

‡ Muddy sand *Zostera* substrate.

bisuturalis occur at low densities relative to the biomass and numbers of their hosts. The percentages derived from the weights in Tables III and IV are all similar, ranging from 0.035 to 0.165 with only the predominant hosts considered and 0.029 to 0.079 when all potential hosts are pooled.

When starved to some degree, *Boonea* species fed on many of the gastropods and bivalves that were offered them (Table V), including not only their probable predominant hosts and other species in the same habitats (Tables III-IV) but even species from other habitats. *B. bisuturalis* fed on a record 37 mollusk species (Table V). It did not feed on the polychaetes *Nereis* (*Neanthes*) *succinea* and *Hydroides dianthus*, the chiton *Chaetopleura apiculata* or the ascidian *Molgula manhattensis*.

Aside from a few interactions that probably were not monitored long enough, the only proffered gastropod and bivalve "hosts" that were not observed even to elicit feeding positions or probing were a large gastropod with a thick integument (*Busyon*), and small species with tight closure (*Caecum*, *Mysella* and *Gemma*). The bivalves *Anadara*, *Anomia*, *Tellina* and *Cumingia* were fed on reluctantly or not at all.

Starved Texas *Boonea* "impressa" was frequently observed to feed cannibalisti-

TABLE III

Weights and numbers of individual mollusks from two 1.0 × 1.0 m quadrats in a habitat where *Boonea seminuda* is relatively abundant on *Crepidula fornicata*, showing the abundance of the *Boonea*.

Species	Quadrat 1 Weight (g)/ [no. individuals]	Quadrat 2 Weight (g)/ [no. individuals]
1. <i>Crepidula fornicata</i> (adults)	256.484 [74]	159.472 [49]
2. <i>Littorina littorea</i> (mainly large adults)	29.874 [15]	6.526 [4]
3. <i>Crassostrea virginica</i> (juvenile and adults)	25.143 [5]	0.531 [1]
4. <i>Bittium alternatum</i> (juveniles)	not weighed or counted*	
5. <i>Anomia simplex</i> (juvenile to medium-sized)	1.221 [21]	3.972 [42]
6. <i>Crepidula convexa</i> (mainly juveniles)	1.187† [78†]	3.228† [140†]
7. <i>Urosalpinx cinerea</i> (adults)	3.707 [2]	0 [0]
8. <i>Crepidula plana</i> (juvenile and adults)	0.477 [2]	0.006 [1]
9. <i>Boonea seminuda</i> (mainly juveniles)	0.090 [24]	0.063 [25]
Totals	318.183 [221]	173.798 [262]
(excluding <i>Bittium</i>)		
Weight of <i>Boonea</i> :		
percent of weight of <i>Crepidula fornicata</i>	0.035	0.040
percent of weight of all "hosts"	0.029	0.037

* Very numerous mainly above the substratum on the red alga *Polysiphonia*; rank estimated.

† Including egg sacs.

‡ Excluding egg sacs.

cally (Table V), unlike the other species. *B. bisuturalis* was once seen to feed on another odostomioid, *Fargoa bartschi* (Table V).

The degree of unselectiveness in feeding differs among the *Boonea* species. *B. "impressa"* (from Texas) fed on 36 out of the 37 gastropod and bivalve species offered to it (97%) (Table V). *B. bisuturalis* fed on 37 out of the 46 gastropod and bivalve species offered to it (80%) (Table V). *B. seminuda* fed on 22 out of the 36 gastropod and bivalve species offered to it (61%) (Table V). *B. seminuda* is more selective than either of the other species, feeding reluctantly or not at all on most neogastropods (*Urosalpinx* through *Ilyanassa* in Table V) and many bivalves.

The "hosts" were pierced in all accessible places (Table V), including tentacles which might be thought too sensitive for this (Ankel and Christensen, 1963). *Melampus* and *Littorina "saxatilis"*, kept submerged with difficulty, were fed on only under water.

Boonea was never seen to feed on individual hosts much smaller than itself. (In the case of *Odostomia*, Ankel and Christensen, 1963, often observed that the parasite was larger than the host.) Only tiny juvenile *B. bisuturalis* were seen to feed on tiny juvenile *Littorina littorea*. The many juveniles of the smaller mollusks encountered in the quadrats (Tables III-IV) no doubt would be immune from parasitism by adult *Boonea*.

In the laboratory, *Fargoa dianthophila* and *F. bartschi* probed and fed only on *Hydroides dianthus*. *F. dianthophila* fed readily both on the collar and on the branchiae. *F. bartschi* was seen to feed (on the collar) only once.

In the experiments on the presumably chemosensory responses of *Boonea* to

two hosts at a distance, *Boonea seminuda* had a total of 234 individuals (77%) on *Crepidula fornicata* and 72 individuals (23%) on *Littorina littorea* in ten trials. The data for *B. seminuda* were found to be sufficiently homogeneous ($0.70 < P < 0.80$) to be treated as a single sample. A Chi-square with one degree of freedom, corrected for continuity, was then performed, resulting in a probability much less than 5 in 10,000 ($P < 0.0005$) that *B. seminuda* was randomly distributed between *C. fornicata* and *L. littorea*. *B. seminuda* showed a clear preference for *C. fornicata*. Boss and Merrill (1965) also demonstrated the same preference.

In the 8 trials of *Boonea bisuturalis* a total of 102 individuals (29%) were on *Crepidula fornicata*, while 246 individuals (71%) were on *Littorina littorea* after 3 hr. The data for *B. bisuturalis* were also tested for homogeneity. These data were found not to be uniform in magnitude, although they all differed in the same direction. The pooled Chi-square was highly significant, but the heterogeneity Chi-square was significant as well. Chi-squares were then computed for each of the eight trials. The Chi-squares for the first three trials are not significant. In the final five trials, after acclimating to laboratory conditions, *B. bisuturalis* showed a clear preference for *L. littorea* ($P < 0.0005$ in trials 4-6; $0.005 > P > 0.001$ in trials 7-8).

DISCUSSION

Boonea seminuda and *B. bisuturalis* occupy different (but slightly overlapping) habitats. Judging by the Woods Hole field data (Tables II-IV), the feeding

TABLE IV

Weights and numbers of individual mollusks (juveniles as well as adults) from one 1.0 × 1.0 m quadrat and one 0.5 × 0.5 m quadrat in a habitat where *Boonea bisuturalis* is relatively abundant near and on *Littorina littorea*, showing the abundance of the *Boonea*.

Species	Quadrat 1 (1.0 × 1.0 m) Weight (g)/ [no. individuals]	Quadrat 2 (0.5 × 0.5 m) Weight (g)/ [no. individuals]
1. <i>Littorina littorea</i>	132.005 [2,156]	68.647 [708]
2. <i>Mercenaria mercenaria</i>	0 [0]	33.997 [2]
3. <i>Mya arenaria</i>	0.219 [7]	25.828 [14]
4. <i>Petricola pholadiformis</i>	0.901 [3]	11.051 [13]
5. <i>Urosalpinx cinerea</i>	20.418 [48]	2.288 [5]
6. <i>Littorina "saxatilis"</i>	1.511 [63]	0.530 [17]
7. <i>Boonea bisuturalis</i>	0.069 [10]	0.113 [24]
8. <i>Crepidula convexa</i>	0.147* [18†]	0 [0]
9. <i>Mitrella lunata</i>	0.021 [3]	0.029 [7]
10. <i>Bittium alternatum</i>	0.046 [5]	0.013 [5]
11. <i>Littorina obtusata</i>	0.001 [1]	0.019 [1]
12. <i>Argopecten irradians</i>	0.017 [2]	0 [0]
13. <i>Anomia simplex</i>	0.006 [1]	0.001 [1]
Totals	155.361 [2,317]	142.516 (797)
Weight of <i>Boonea</i> :		
percent of weight of <i>Littorina littorea</i>	0.052	0.165
percent of weight of all "hosts"	0.044	0.079

* Including one cluster of egg sacs.

† Excluding one cluster of egg sacs.

TABLE V

Systematic list of mollusks offered to *Boonea seminuda* (from Woods Hole), *B. bisuturalis* (from Woods Hole), and *B. "impressa"* (from Galveston) in bowls in the laboratory, and the feeding interactions observed. Occurrence (habitat coincidence) is considered in terms of the likelihood of contact. Abbreviations: A—absent; AS—anterior siphon; BES—base of excurrent siphon; C—common; CNO—cannibalism not observed; DIMC—deep in mantle cavity; EDIFE—edge and deep in from edge; F—foot; FEF—front end of foot; FFRD—fed on fairly readily; FFRU—fed on fairly reluctantly; FRD—fed on readily; FRL—fed on reluctantly; IC—inaccessible; IFE—in from edge; IP—impenetrable; IS—incurrent siphon; LIT—large and integument thick; M—mantle; MBTS—mantle between two siphons; ME—mantle edge; MES—mantle through excurrent siphon; MF—mantle fusion; NMLE—not monitored long enough; OL—opercularious lobe; PH—predominant host; R—rare; RC—reluctant cannibalism; RF—rear end of foot; RH—rear of head; SA—siphonal area; SBF—side and base of foot; SF—side of foot; SH—side of head; STC—small and tight closing; T—tentacle; TIES—through incurrent and excurrent siphon; U—unpalatable; USF—under side of foot.

Taxa	<i>Boonea seminuda</i>					<i>Boonea bisuturalis</i>					<i>Boonea "impressa"</i>				
	Occurrence	Feeding position	Probing	Feeding	Puncture site	Remarks	Occurrence	Feeding position	Probing	Feeding	Puncture site	Remarks	Feeding position	Probing	Remarks
Polyplacophora															
<i>Chaptalia opiculata</i>	A	+	+	+	M (EDIFE) ME; SBF ME	FFRD FFRD FFRD FFRL	R	+	+	+	—	U or IC PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
Gastropoda															
<i>Lacuna vincla</i>	C	+	+	+	T	FRD	A	+	+	+	DIMC SF; ME; OL DIMC	PH; FRD PH; FRD FFRL FFRL	+	+	SF ME; OL T ME
<i>Littorina littorea</i>	A	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Littorina obtusata</i>	A	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Littorina saxatilis</i>	A	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Littorina viridis</i>	A	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Gyrodont cf. j. talleni</i>	A	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Gyrodont cf. j. talleni</i>	A	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Bitium alternatum</i>	C	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Cerithiopsis emersoni</i>	C	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Sella adamsi</i>	R	+	+	+	—	FFRL	C	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Crepidula fornicata</i>	R	+	+	+	—	FFRL	R	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Crepidula comexa</i>	C	+	+	+	—	FFRL	R	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Crepidula plana</i>	R	+	+	+	—	FFRL	C	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Vrosalpinx cinerea</i>	R	+	+	+	—	FFRL	C	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Ensis lapidaria</i>	A	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Ensis armatus</i>	A	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Anachis latirayayi</i>	A	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Anachis anara</i>	A	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Murella lunata</i>	A	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Busycan conatulaum</i>	A	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Nassarius vibex</i>	A	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Nassarius trivittatus</i>	A	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Hydrobia ulitoides</i>	A	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Boonea seminuda</i>	C	+	+	+	—	FFRL	A	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD
<i>Boonea bisuturalis</i>	C	+	+	+	—	FFRL	C	+	+	+	—	PH; FRD PH; FRD FFRL FFRL	+	+	FFRD FFRD FFRD FFRD

TABLE V—Continued

Taxa	Boonea seminuda					Boonea bisuturalis					Boonea "impressa"					Remarks		
	Occurrence	Feeding position	Probing	Feeding	Puncture site	Remarks	Occurrence	Feeding position	Probing	Feeding	Puncture site	Remarks	Occurrence	Feeding position	Probing		Feeding	Puncture site
Boonea "impressa"	A	+	+	+	ME?; DIMC	FFRL	R	+	+	+	+	FFRL	C	+	+	+	FEF	RC
Fargoa barischi	A	+	+	+	ME	FFRL	A	+	+	+	+	FFRL	A	+	+	+	T	FFRD
Metampyx bidentatus	A	+	+	+	—	FFRL	A	+	+	+	+	FFRL	A	+	+	+	ME	FFRD
Bivalvia	A	+	+	+	—	U	R	+	+	+	+	FFRL	A	+	+	+	ME	FFRD
Solenus velum	A	+	+	+	—	U	R	+	+	+	+	FFRL	A	+	+	+	ME	FFRD
Anadara transversa	A	+	+	+	—	FFRD	R†	+	+	+	+	FFRD	A	+	+	+	ME	FFRD
Anadara ovalis	A	+	+	+	M (IFE)	FFRD	R†	+	+	+	+	FFRD	A	+	+	+	ME	FFRD
Mytilus edulis	A	+	+	+	—	FFRD	R	+	+	+	+	FFRD	C	+	+	+	ME	FFRD
Brachiodontes exustus	A	+	+	+	—	FFRD	R	+	+	+	+	FFRD	C	+	+	+	ME	FFRD
Ischadium recurvum	A	+	+	+	—	FFRD	R	+	+	+	+	FFRD	C	+	+	+	ME	FFRD
Gemma gemma	A	+	+	+	—	FFRD	R	+	+	+	+	FFRD	C	+	+	+	ME	FFRD
Argopecten irradians	A	+	+	+	—	FFRD	R	+	+	+	+	FFRD	C	+	+	+	ME	FFRD
Anomia simplex	A	+	+	+	—	FFRD	R	+	+	+	+	FFRD	C	+	+	+	ME	PH,FRD
Grossostrea virginica	A	+	+	+	—	FFRD	R	+	+	+	+	FFRD	C	+	+	+	ME	PH,FRD
Mytilus planulata	A	+	+	+	—	U	A	+	+	+	+	STC	A	+	+	+	ME	FFRL
Laevicardium mortoni	A	+	+	+	—	U	A	+	+	+	+	FFRD	A	+	+	+	ME	FFRD
Spisula solidissima	A	+	+	+	—	U	A†	+	+	+	+	FFRD	A†	+	+	+	SA	FFRD
Mulinia lateralis	A	+	+	+	—	U	A	+	+	+	+	MBTS	A	+	+	+	SA	FFRD
Ensis directus	A	+	+	+	—	U	A	+	+	+	+	—	A	+	+	+	—	FFRL
Tellina agilis	A	+	+	+	—	U	A	+	+	+	+	—	A	+	+	+	—	FFRL
Cumingia tellinoides	A	+	+	+	—	U	A	+	+	+	+	—	A	+	+	+	—	FFRD
Mulinia tencipallata	A	+	+	+	—	U	A	+	+	+	+	—	A	+	+	+	—	FFRD
Mercenaria mercenaria	A	+	+	+	—	U	A	+	+	+	+	—	A	+	+	+	—	FFRL
Gemma gemma	A	+	+	+	—	U	A	+	+	+	+	—	A	+	+	+	—	FFRL
Patella phaladiformis	A	+	+	+	—	FFRL	R	+	+	+	+	FFRL	C	+	+	+	IS	FFRL
Mya arenaria	A	+	+	+	—	U	C	+	+	+	+	STC	C	+	+	+	MBTS	FFRL
Lyonsia hyalina	A	+	+	+	—	FFRL	C	+	+	+	+	FFRL	A	+	+	+	BES	FFRD
Totals		32	27	22			A	40	40	37			37	36	36			

* At Woods Hole; association common at Plymouth, MA.

† Juvenile host used.

experiments (Table V) and the experiments on the attractiveness of different hosts, *B. seminuda* clearly feeds primarily on *Crepidula fornicata* and *B. bisuturalis* primarily on *Littorina littorea*. Thus the two *Boonea* niches differ in respect to preferred habitat and predominant host, and competition for space and food is largely avoided.

Assuming that what happens in the laboratory also happens in nature and that preferences for the predominant hosts are not overriding, it seems probable that some of the other mollusk species living in the same habitats serve as natural secondary hosts for *Boonea*. Leaving aside *Crepidula fornicata*, *B. seminuda* occurs with five other common mollusk species; four of these were fed on in the laboratory (Table V). Four rare species co-occur, and three of these were also fed on in the laboratory (Table V). Leaving aside *Littorina littorea*, *B. bisuturalis* occurs with seven other common species; all of these were fed on in the laboratory (Table V). Fifteen rare species co-occur, and all but two of these were also fed on in the laboratory (Table V). Rarely, *B. seminuda* is associated with *Littorina littorea* in the field (Table II) and hence rather definitely feeds on it in nature. The same holds for *B. bisuturalis* with *Crepidula fornicata* (Table II). Predominant hosts are thus reversed occasionally. Leaving aside *Crassostrea virginica*, *B. "impressa"* occurs with six other common species; all of these were fed on in the laboratory (Table V). Of these, *Geukensia demissa* and the *Ischadium* appear in Table II and hence are particularly likely natural secondary hosts.

Molgula and an unidentified polychaete are the only non-molluscan hosts reported to be fed on by a *Boonea* (Allen, 1958; *B. impressa*). *Molgula* was not fed on by *B. bisuturalis* despite intermittent monitoring for three weeks. *B. bisuturalis* sometimes assumed a feeding position on either of the siphons, just as it does on bivalves. Probing into a siphon was seen once. Allen (1958) may also have seen probing. The tunic of *Molgula* is probably too thick and tough for penetration by a *Boonea* proboscis. The cuticle of *Nereis* is probably also impenetrable.

Boonea clearly is a generalist molluscivore, by no means being host-specific. If some selective catastrophe befell any of the predominant hosts, the *Boonea* populations no doubt would be greatly reduced but would survive on other molluscan hosts.

Boonea bisuturalis is an indigenous species at Woods Hole, having been described (as *Turritella bisuturalis*) by Say (1822) with "Boston harbour" as the type-locality. *Littorina littorea*, on the other hand, is introduced and has been at Woods Hole only since 1875 (Wells, 1965). (This in itself indicates that *B. bisuturalis* is not host-specific.) It would be interesting to know the host or hosts of *B. bisuturalis* before this introduction. Assuming that there has been no habitat change, *Mya arenaria* (see Medcof, 1948), *Petricola pholadiformis*, *Urosalpinx cinerea* and *Mercenaria mercenaria* are possibilities. Note also that at Plymouth, *Ilyanassa obsoleta* was a common substrate (Table II) in a mud habitat. *Crassostrea virginica*, the predominant host of *B. impressa* (which hybridizes or intergrades clinally with *B. bisuturalis* in the New York-New Jersey area), was never found to be a living substrate for *B. bisuturalis* at Woods Hole. *Crassostrea* was, however, reported as a natural host elsewhere in New England by Loosanoff (1956) and Boss and Merrill (1965).

In terms of biomass, *Crepidula fornicata* and *Littorina littorea*, the predominant hosts of *Boonea seminuda* and *B. bisuturalis*, are dominant in their respective habitats (Tables III and IV). *L. littorea* is also dominant numerically. The low densities of the two *Boonea* species relative to the biomass and numbers of their hosts (Tables III and IV) make it likely that these odostomioids do little harm to their hosts—injuries such as reported by Cole and Hancock (1955), Loosanoff (1956), and Allen (1958) were not observed. Judging by the four quadrats and the two species, a given biomass of host may support a relatively stable percentage biomass of *Boonea*. Note that the percentages derived from the weights in Tables III and IV are all similar.

The anomalous results of the experiments with *B. bisuturalis* are probably due to the individuals having just been collected. The trials with *B. seminuda* started after these animals had been in the laboratory for two weeks feeding on *Crepidula fornicata* and *Littorina littorea*. Something caused the *B. bisuturalis* to move randomly in their first 48 hr in the laboratory. Possibly they were disoriented or hungry.

In contrast to the pair of *Boonea* species at Woods Hole, the pair of *Fargoa* species is in direct competition with each other for space and food (*Hydroides dianthus*). They occasionally exist even on the same individual worm. *F. dianthophila* is the commoner and smaller of the two species. Sometimes it rides in and out of the worm's tube while sitting on the worm's operculum. At other times it sits on the tube. *F. bartschi* never enters the tube since (at least when adult) it is too long to turn around.

In the Woods Hole area *Hydroides dianthus* is sparsely and patchily dispersed, and spatfalls vary in success at each locality from year to year (personal observations). Not surprisingly, the *Fargoa* populations are commensurately sparse, patchy and sporadic. *Hydroides dianthus* is sympatric with other species of *Hydroides* (*Eupomatus* is a synonym) off North Carolina, northeast and north-west Florida. *Fargoa dianthophila* occurs with three of these other *Hydroides* species (Wells and Wells, 1969). The same is probably true of *F. bartschi*, which ranges from Massachusetts to Texas. Thus the two *Fargoa* species seem both to be specialists host-specific to the genus *Hydroides*. This occurrence of more than one odostomioid on the same serpulid is reminiscent of *Odostomia plicata*, *O. lukisi*, *O. unidentata* and "*Chrysallida*" (*Partulida*) *spiralis* of Europe, all of which parasitize *Pomatoceros triqueter* (Ankel, 1959; Fretter and Graham, 1962).

Boonea is a generalist and *Fargoa* a specialist. It seems likely that the former condition is the more primitive, and that pyramidellids were not originally host-specific.

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SUMMARY

1. Three *Boonca* species (occurring in sympatric species pairs) occupy different habitats and have different molluscan host preferences. In the field, *B. seminuda* is preferentially with *Crepidula fornicata* or *Argopecten irradians*, *B. bisuturalis* with *Littorina littorea* (introduced), *Ilyanassa obsoleta* or *Crassostrea virginica*, and *B. impressa* with *C. virginica*. Weights of the first two species are about 0.03 to 0.17% those of their hosts. In the laboratory, *B. seminuda* was attracted much more to *Crepidula fornicata* than to *Littorina littorea*. With *B. bisuturalis* it was vice versa.

2. In the laboratory, *B. seminuda* fed on 22 out of the 36 gastropod and bivalve "hosts" offered; *B. bisuturalis* fed on 37 out of 45, and *B. impressa* fed on 36 out of 37. Some of these mollusks probably serve as secondary hosts in nature. *Boonca* definitely is not host-specific. Polychaetes, *Chaetopleura* and *Molgula* were not fed on.

3. *Fargoa dianthophila* and *F. bartschi*, two much rarer species, compete with each other for space and food by both obligately parasitizing species in the genus *Hydroides*, sometimes co-occurring on the same individual. *Fargoa* species do seem to be host-specific.

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FIELD AND LABORATORY STUDIES OF *GLUGEA HERTWIGI*
(MICROSPORIDA) IN THE RAINBOW SMELT
*OSMERUS MORDAX*¹

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Glugea hertwigi-induced microsporidiosis is a disease of the smelt, *Osmerus mordax*. *O. mordax* is an anadromous species that has been successfully introduced to temperate freshwater areas. Haley (1957) has provided some evidence which indicates *G. hertwigi* was in part responsible for the decline of the smelt fishery in the Atlantic. The incidence of *Glugea* infection reaches a seasonal peak of nearly 90% in juvenile Lake Erie smelt each summer and fall; Nepszy, Budd, and Dechtiar (1978) estimate vast economic losses in the smelt fishery in the Great Lakes due to mortality of infected juveniles.

G. hertwigi infections typically localize as parasite colonies in the submucosal layer of the intestine. Mortality of the smelt host is believed to occur by starvation; in addition, infected fish have navigation problems, are more susceptible to predation, and less able to recover from environmental stress (Legault and Delisle, 1967; Nepszy and Dechtiar, 1972). Spawning female smelt in Lake Erie characteristically have numerous *G. hertwigi* cysts in the ovaries as well as along the intestine. Chen and Power (1972) reported a 42% decrease in fecundity of *Glugea* infected females. The microsporidan *Plistophora ovariae* infecting the golden shiner *Notemigonus crysoleucas* is an example of transovarian parasite transmission (Summerfelt and Warner, 1970).

G. hertwigi is an obligate intracellular parasite completing its life cycle within a single host cell. After ingestion by a smelt, the infective spore is believed to discharge a polar filament with a velocity suitable for penetrating the intestinal mucosal cell layer (Ishihara, 1968; Weidner, 1972, 1976). The vegetative stages of the parasite do not cause host cell degeneration but stimulate hypertrophy and abnormal development into a "xenoma" (Weissenberg, 1968, 1976; Weidner, 1974). "Xenoma" refers to the unique association between an hypertrophied host cell and developing intracellular parasites (Weissenberg, 1968, 1976). The host-cell component is induced to undergo extensive growth during vegetative development (schizogony) by the parasite. Eventually the parasite differentiates into mature spores (sporogony) which fill the central region of the xenoma. By this stage, a combination of host animal response and parasite growth transform the xenoma into a thick-walled "Glugea-cyst" filled with innumerable spores (Sprague and Vernick, 1968; Weissenberg, 1968, 1976; Weidner, 1973, 1976). *G. hertwigi* cysts range from 0.4 to 5.0 mm in diameter; heavy smelt infections

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number over 200 xenomas per host (Legault and Delisle, 1967; Nepszy and Dechtiar, 1972).

McVicar (1975), Olson (1976) and Weissenberg (1968) transmitted *Glugea* species to fish held in the laboratory. Weidner (1973) and Stunkard and Lux (1965) suggested that invertebrate filter-feeders may serve as natural vectors or transport hosts for fish microsporidians. Olson (1976) determined a low level of *Glugea stephani* infection occurred by ingestion of spores directly. McVicar (1975) transmitted *G. stephani* through spore-carrying vectors and by injection of spores into the peritoneal cavity of adult fish. Weissenberg (1968) did not determine whether *G. anomala* was initiated by a spore-carrying vector or by direct ingestion of the spores.

We thought it would be of interest to examine *G. hertwigi* growth and multiplication in ovaries of spawning female smelt; follow transmission of infections; and examine the microstructure of early xenoma growth.

MATERIALS AND METHODS

Adult smelt

Spawning female smelt were collected from Wheatley, Ontario, on Lake Erie and from the Jones River, near Plymouth, Cape Cod, Massachusetts. A total of 150 fish from each location were examined internally for the presence of *Glugea hertwigi* cysts. The intestine and ovaries from infected and non-infected fish were excised, cut into small pieces and fixed in pH 7.4 phosphate buffered glutaraldehyde overnight at 4° C. After several buffer rinses, the material was post-fixed in phosphate buffered 2% osmium tetroxide for 2 hr at 4° C, dehydrated in ethanol and embedded in Epon. One micron sections were cut on a Dupont-Sorvall MT-2B microtome and stained with 1% toluidine blue. Parasite cysts were removed from adult smelt, homogenized, pelleted in pH 7.4 phosphate buffer, stored at 4° C and used for the transmission experiments.

Transmission experiments

Eggs and milt were stripped from spawning smelt, mixed 1:5 respectively and kept in a well-aerated nylon mesh cone. Naturally fertilized eggs were also collected from the river-bed. Anticipating large mortalities, a non-infected smelt population was located in Long Pond, Cape Cod, Massachusetts. Several years' examination of smelt from this pond had indicated they were completely free of *G. hertwigi* infection. Young smelt (20–25 mm) in Long Pond were attracted at night to a strong light at the surface and collected by hand-net. All fish were maintained at the National Marine Fisheries Service Aquarium, Woods Hole, in 20° C filtered fresh water taken from the Jones River well above the spawning sites. All fish were maintained on a diet of phyto- and zooplankton seined from Long Pond. The following methods of parasite transmission were attempted.

Experiment 1. Laboratory reared and collected smelt were exposed to a suspension of *G. hertwigi* spores placed in the tanks. The water was well aerated but not filtered for the following 48 hr.



FIGURE 1. Young specimen of the smelt, *Osmerus mordax*, experimentally infected with *Glugea hertwigi*. The yearly incidence of natural infections nears 90% in juveniles with consequent vast mortalities. Seven days post infection, xenomas (white arrows) develop proximate to larger 4-week-old "Glugea cysts" (black arrows). Bar represents 2.5 mm.

Experiment 2. Laboratory reared smelt (10 mm, 6 weeks post spawning) were fed spore-carrying zooplankton (cladocerans and copepods) on 2 consecutive days. The plankton was first exposed to a spore suspension for 30 min, washed once with water, examined to ensure the presence of spores in their digestive tracts and then fed to the smelt.

Experiment 3. Collected smelt (15 mm, approximately 8 weeks post spawning) were fed spore-carrying plankton as above.

Experiment 4. Smelt from Experiment 1, 4 weeks after exposure to a spore suspension, were fed spore-carrying plankton as above. As a control, a number of laboratory reared and collected smelt were maintained unexposed to spores.

Development of xenomas

Young smelt from all tanks were observed and photographed with a Wild M-4 Makroskope 5 days, 1, 2 and 3 weeks after spore feeding. Intestinal tissue from experimental and control smelt were prepared for microscopy as outlined above.

TABLE I

Experiment number	Source of smelt _j	Sample size	Method of exposure	Period of incubation	Number of smelt infected	
					Light < 5 (per host)	Heavy > 50 (per host)
1	mixture of lab reared and collected	20	spore suspension	28 days	8	0
2	lab reared	5	spore-carrying plankton	7 days	0	5
3	collected	14	spore-carrying plankton	7 days	0	14
4*	mixture	8	spore-carrying plankton	7 days	0	8

* Smelt from Experiment 1, carrying light infections 28 days after exposure to the spore suspension.

RESULTS

Adult smelt

In spawning females *Gluga hertwigi* cysts were found in the ovaries of more than 50% of the infected fish in Lake Erie and in 25% of infected fish in the Jones River. The ovaries of Lake Erie fish were heavily infected whereas those of the Jones River were lightly infected. Sections of infected ovaries showed the parasite was isolated from the scattered ova by the cyst wall. No free spores or developing stages of *G. hertwigi* were observed outside the cysts, in ova or in ovarian tissue.

Parasite transmission

The results of the experiments are summarized in Table I. Positive results were obtained from both methods of spore transmission; however, the intensity of infection and its effect differed.

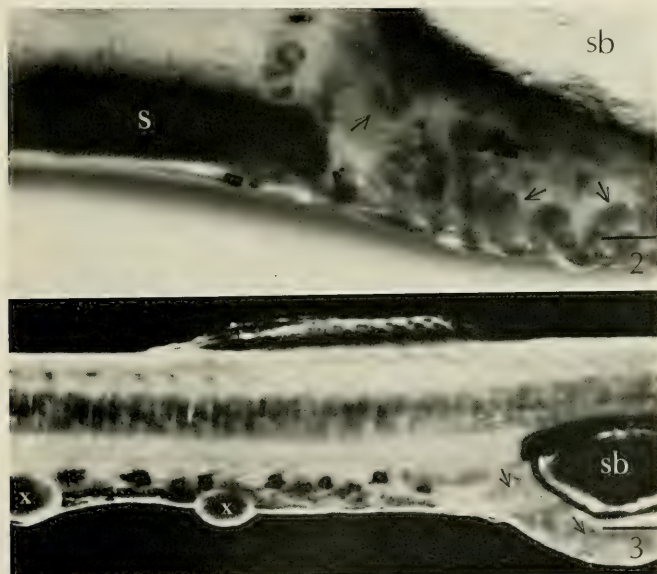


FIGURE 2. Young smelt, 12 days after feeding on spore-carrying plankton. Numerous xenomas (arrows) begin just behind the stomach and continue along the gut to the vent. S, stomach; sb, swimbladder. Bar represents 180 μ m.

FIGURE 3. Young smelt previously infected after exposure to a spore suspension developed numerous new xenomas (arrows) along the intestine when fed spore-carrying plankton. X, xenomas from exposure to spores directly. Bar represents 750 μ m.

Experiment 1. Laboratory-reared and collected fish were exposed to a suspension of *G. hertwigi* spores. Four weeks after exposure, 40% of the smelt exhibited one or two *Glugea* cysts in the posterior region of the intestine. These cysts were uniformly dense and protruded from the submucosa distending the peritoneal cavity. The fish fed continually throughout the experimental period. Several fish died during the period, were examined and one was found infected with a single *Glugea* cyst.

Experiment 2. Laboratory-reared fish were exposed to spore-carrying plankton. One week after exposure all fish exhibited numerous small xenomas beginning just behind the stomach and continuing along the entire length of the intestine to the vent (Fig. 2). Gradually the fish stopped feeding, had difficulty swimming and all died by 16 days after exposure to the spores.

Experiment 3. Collected smelt were exposed to spore-carrying plankton. The results were similar to those in Experiment 2; however, these fish stopped feeding and died at about 25 days after exposure to the parasite.

Experiment 4. Smelt from Experiment 1, 4 weeks after being exposed to a spore suspension, were fed spore-carrying plankton. One week after feeding, all fish exhibited numerous small xenomas along the intestine as well as the large posterior xenomas (Fig. 3). All fish died within 2 weeks after exposure to the spore-carrying vectors. Controls were examined periodically and found free of infection.

Parasite development

Parasite growth was rapid at 20° C. Examination of the intestine from a heavy infection (Experiments 2, 3, and 4) showed the extensive tissue involvement. Infections protruded to the serosa and were easily dissected away intact (Fig. 4). Extensive host cell hypertrophy was the obvious feature of sectioned material (Fig. 5). Xenomas ranged from 20 to 50 μ m in diameter. Smaller xenomas contained one or two greatly enlarged host cell nuclei; whereas the larger xenomas

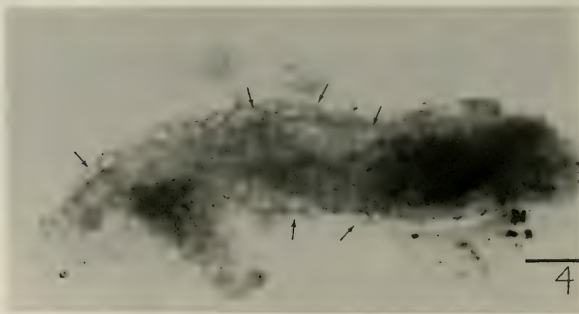


FIGURE 4. Whole intestine 1 week after exposure to spore-carrying plankton. Xenomas (arrows) protrude to the serosa and are easily dissected away intact. Bar represents 80 μ m.

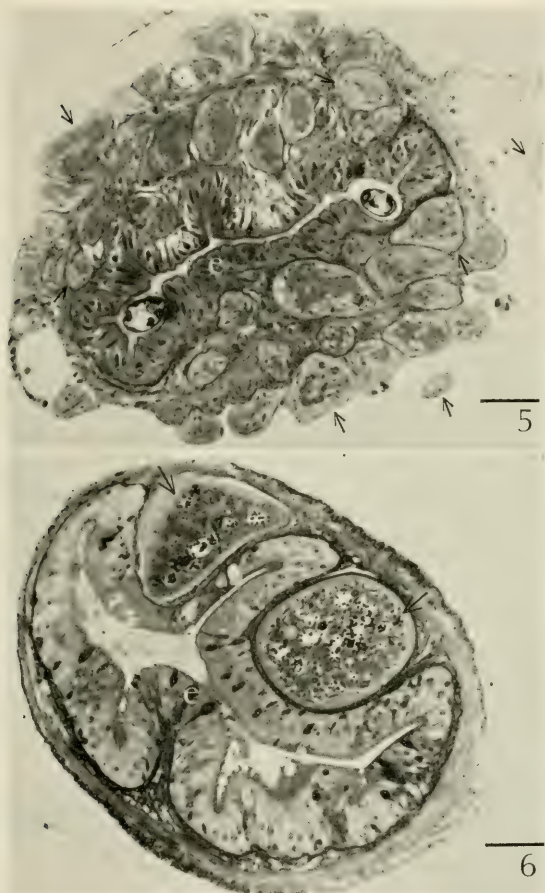


FIGURE 5. Cross section of intestine similar to that shown in Figure 4. At least 40 xenomas (arrows) 20 to 50 μm in diameter develop within the submucosa and protrude from the mucosa. Infected host cells hypertrophy and contain early schizont stages of the parasite, e, epithelium. One micron Epon section; 1% toluidine blue stain; bar represents 50 μm .

FIGURE 6. Cross section of intestine from lightly infected smelt 2 weeks after feeding on a spore suspension. Progressive hypertrophy increased the xenomas to 100 to 125 μm in diameter. Xenomas develop in the submucosal layer causing mechanical distension of the epithelium (e). Sporogony stages and free dense spores fill the central region of the xenoma. One micron Epon section; 1% toluidine blue stain; bar represents 50 μm .

were multinucleated with many nuclei lobed or branched, indicating nuclear division. At 1 week the parasite formed schizont colonies peripheral to the host cell nuclei. After 2 weeks of growth there was considerable increase in host cell hypertrophy and *Glugea* maturation to spores. Xenomas ranged from 100 to 125 μm in diameter with host cell nuclei, cytoplasmic components and *G. hertwigi* schizonts particularly obvious in the peripheral region of the xenomas; sporoblastic stages of the parasite were common in the central region (Fig. 5).

DISCUSSION

Transovarian parasite transmission is known from a number of microsporidan species (Kudo, 1966). Recently, Summerfelt and Warner (1970) demonstrated a *Plistophora ovariae* infection in viable eggs of the golden shiner, *Notemigonus crysoleucas*. Although the ovaries of spawning female smelt were often loaded with *G. hertwigi* parasites, our thorough examinations indicate *G. hertwigi* are not present in germinative or egg cells. Previously, there has been limited success in experimental peroral transmission of fish microsporidians (Delisle, 1969; McVicar, 1975; Summerfelt and Warner, 1970; Stunkard and Lux, 1965). However, Olson (1976) successfully transmitted *G. stephani* to the English sole, *Paraphrys vetulus* in water temperatures above 15° C. Several authors are convinced that transport vectors are necessary to concentrate *Glugea* spores for natural transmission of certain fish microsporidiosis (Haley, 1957; Putz and McLaughlin, 1970; Stunkard and Lux, 1965). Small filter-feeding animals may serve as transport hosts and in addition, may stimulate the spores to hatch and infect the fish. Weidner (unpublished observations) has observed such a phenomenon with *G. stephani* in the winter flounder, *Pseudopleuronectes americanus*. In this study, transmission of *G. hertwigi* to both laboratory-reared and collected smelt was successful at 20° C, either by direct spore consumption or ingestion of spore-carrying vectors; however, a major magnitude of difference exists in the intensity between direct spore and vector transmitted infections. Vector transmission produced massive infections along the entire intestine in all test subjects.

Smelt are selective predators, taking cyclopoid and calanoid copepods and several species of cladocerans as their first food (Reif and Tappa, 1966; Siefert, 1972). Presumably, the natural *G. hertwigi* infection occurs through the ingestion of spore-carrying filter feeders by very young smelt. Release of spores from infected adult smelt occurs via two routes. Scarborough (unpublished observations) has observed the expulsion of parasite xenomas from ovaries during spawning. In this manner, female adult smelt may concentrate *G. hertwigi* spores in the immediate vicinity of developing young. Further, infected smelt carrion were seen being preyed upon by small crustaceans in the nursery areas after spawning. Nepszy and Dechtiar (1972) found that heavily infected adult smelt were unable to recover from spawning stress; mass mortalities in the spawning grounds consisted of infected adults.

Massive infections of *G. hertwigi* consequent to ingestion of spore vectors are fatal to both collected and laboratory reared smelt. Mechanical distention of the intestinal tissue and starvation are thought to be the cause of death. *Osmerus mordax* is a difficult species to raise in the laboratory and the minimal condition

of the reared fish probably precluded a greater susceptibility to the effects of multiple infections. Young smelt tolerate light infections for at least several weeks and likely carry them into adulthood. Nepszy and Dechtiar (1972) found that *G. hertwigi* colonies can remain in smelt for much of the host's life, and stress will significantly increase the mortality rate in these fish over uninfected smelt.

It is well documented that the life cycle of microsporidians begins with injection of the sporoplasm through a spore tube into the host (Vavra, 1976; Weidner, 1972). It is assumed that the discharging tube of *G. hertwigi* spores penetrates through the gut basal lamina delivering the parasite into submucosa cells.

Whether transmitted directly by spores or via spore-carrying vectors, development of *G. hertwigi* was identical, and paralleled that described for other *Glugea* species (Sprague and Vernick, 1968; Weissenberg, 1968, 1976). Changes in macroscopic appearance of xenomas correlated with microscopic examinations of the sectioned material. While the host cell component of the xenoma remains viable, the parasite develops numerous schizonts within the peripheral cytoplasm. The host cytoplasm and nucleus hypertrophy in apparent response to the parasite's presence; subsequently, the nucleus undergoes an amitotic budding. As the xenoma size increases, a cellular capsule delimits the xenoma from the surrounding tissue; this capsule becomes enveloped by host connective tissue layers. While maturation of the parasite progresses through sporogony, host cell components begin to degenerate and spores fill the interior. The cyst stage consists of spores and scattered vestiges of host cell components surrounded by a wall.

Although Weissenberg (1976) believed the host cell to be a presumptive macrophage, the cell type which can support *G. hertwigi* remains undetermined. Our observations indicate massive infections will produce xenomas easily separated from the intestine. Xenomas were observed associated with various visceral organs, including cells below the peritoneal lining. It is not known how *G. hertwigi* enters ovarian tissue; presumably, initially infected host cells enter the blood stream and are delivered to favorable environs for growth, such as the highly vascularized ovaries. *Nosema michaelis*, a microsporidian infecting the blue crab, *Callinectes sapidus*, undergoes vegetative growth in the gut wall and these cells subsequently circulate to muscle tissue for continued development (Weidner, 1972).

This particular host-parasite association likely will be of some use in the study of xenomas. The transparency of young smelt lends itself to *in vivo* study of drug effects on parasite infection and xenoma development. Tissue culture techniques may now be carried out on isolated xenomas since these larger tumor cells are readily detectable in young smelt and easily removed aseptically from the gut serosa.

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SUMMARY

Glugea hertwigi-induced microsporidiosis is a disease of the smelt *Osmerus mordax*. The yearly incidence of infection reaches over 50% in adult smelt and as

high as 90% in juveniles. Primary infections localize as large intracellular colonies in submucosal cells of the digestive tract. Field observations indicate the ovaries of spawning females are the secondary site of infection. *G. hertwigi* was successfully transmitted to both laboratory-reared and collected young smelt at 20° C by small filter-feeding vectors and by direct ingestion of spores. Infections transmitted by spore-carrying vectors numbered hundreds per animal, and were visible along the intestine one week after feeding. Large parasitized host-cells (xenomas) extended from the intestinal serosa and were easily recovered. *G. hertwigi* infections acquired by direct spore feeding numbered one or two per animal; these fish have the capacity to develop many new infections by feeding on spore-carrying vectors. Microscopic study revealed that *G. hertwigi* development was indeed within a single greatly hypertrophied host cell. After 1 week of growth, 20 to 50- μ m xenomas contained a few enlarged host nuclei and vegetative *G. hertwigi*; after 2 weeks, the xenomas measured 100 to 125 μ m, exhibited multiple host nuclei and numerous *G. hertwigi* sporoblasts and spores.

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DEVELOPMENT OF TAIL MUSCLE ACETYLCHOLINESTERASE IN ASCIDIAN EMBRYOS LACKING MITOCHONDRIAL LOCALIZATION AND SEGREGATION

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Ascidians (subphylum Urochordata; class Ascidiacea) develop a large number of mitochondria during oogenesis. At fertilization many of these become localized into a myoplasmic crescent and are subsequently segregated by the determinate cleavage mechanism into the muscle lineage cells of the developing larva. In some species colored yolk granules are associated with the mitochondria; they serve as visible markers of the mitochondrial localization and its segregation (Conklin, 1905; Berrill, 1929; Berg and Humphreys, 1960). Many different staining techniques have been used to establish that oviparous species have mitochondrial segregation even in the absence of a visible crescent (*e.g.*, Meves, 1913; Duesberg, 1915; Mancuso, 1952; Reverberi, 1956).

This obvious association of large numbers of mitochondria with the ascidian larval muscle cell lineage has raised questions about whether the crescent mitochondria are (a) permissive, (b) selective, or even (c) instructive of muscle differentiation. An equally important question is whether segregation of the cytoplasmic determinants responsible for muscle differentiation (Whittaker, 1973) is linked directly to the mechanisms that localize and segregate mitochondria into the muscle cells. The results of various observations and experiments that have attempted to answer these questions prove to be contradictory. The suggestion has therefore persisted in some of the review literature that mitochondrial localizations are causally related to muscle cell determination and development (Brachet, 1960, 1974; Minganti, 1961; Reverberi, 1961, 1971).

Conklin (1931) displaced mitochondria by centrifugation of unfertilized ascidian eggs and concluded from his results that localizations of mitochondria were not the cause of muscle determination. Mitochondria could be driven out of the finely granular plasm in which they were found without preventing the formation of muscle during subsequent development. When the plasm itself was displaced, the larval muscles were also displaced. Tung, Ku, and Tung (1941) noted that mitochondrial masses moved by centrifugation to neural and ectodermal regions did not cause these cells to develop myofibrillae.

The first contradictory evidence came from centrifugation experiments by Ries (1939) who found that displacement of the indophenol oxidase-containing plasm of the ascidian egg resulted in a change in muscle development; he did not know at the time that this enzyme was a mitochondrial oxidase. La Spina (1958) also showed that mitochondrial displacement resulted in some abnormalities of muscle development. Direct interference with mitochondrial function, using inhibitors of the mitochondrial enzymes, resulted in the development of embryos markedly

deficient in muscle structures (de Vincentiis, 1956; Reverberi, 1957). Recently, Bell and Holland (1974) have found by microsurgically dividing centrifugally stratified eggs in various orientations and fertilizing the fragments that a certain limited number of mitochondria appear to be necessary for muscle differentiation. Their data also confirm the possibility that mitochondrial localizations might be causally related to muscle differentiation.

One of the difficulties with these various traumatic and disruptive experimental interventions is that they cause severe abnormalities in the embryos. Results are subject to considerable selection and interpretation by the investigator. In nature, however, a situation occurs in which the question can be clearly resolved in a qualitative way. Certain ascidian species have secondarily evolved anural larvae which no longer develop the larval tail (Berrill, 1931). One of these species, *Molgula arenata*, differentiates larval tail muscle up to the point of producing histospecific muscle acetylcholinesterase (Whittaker, 1979). This species does not localize mitochondria or segregate them into the muscle lineage cells.

MATERIALS AND METHODS

Materials

The specimens of *Molgula arenata* Stimpson used in this study were dredged from sand flats near Senator Shoal in northern Nantucket Sound at Cape Cod, June through November. Control observations were made on embryos of two urodele species: *Ciona intestinalis* (L.), obtained in the vicinity of Woods Hole, Massachusetts, and *Molgula occidentalis* Traustedt of the Florida Gulf, purchased from the Gulf Specimen Company, Panacea, Florida. These three species do not have a visibly colored mitochondrial crescent.

Gametes were obtained by techniques described previously (Whittaker, 1979). Embryos were cultured in filtered sea water at $18 \pm 0.1^\circ \text{C}$ using a refrigerated constant temperature water bath. Since the time of first division is variable in *M. arenata* (Whittaker, 1979), development times are expressed as time after the 2-cell stage.

Histochemistry

Acetylcholinesterase was localized in embryos by the Karnovsky and Roots (1964) procedure after 2 to 3 min fixation in cold (5°C) 80% ethanol (Durante, 1956). Incubation was for 12 hr at 18°C . Various substrate and inhibitor controls for the identity of the enzyme are presented elsewhere (Whittaker, 1979).

Succinic dehydrogenase activity was detected by the standard technique for bound mitochondrial enzymes described by Pearse (1972) using sodium succinate as substrate and nitro blue tetrazolium as the electron acceptor. Cytochrome oxidase was localized by the diaminobenzidine reaction of Seligman, Karnovsky, Wasserkrug, and Hanker (1968) including 20 $\mu\text{g}/\text{ml}$ Sigma C-40 catalase in the reaction medium to prevent peroxidase activity. The fixative used for both enzymes was Karnovsky's (1965) fixative, but with formaldehyde and glutaraldehyde each reduced to 1.5%, half of the originally recommended strength. Fixation was for 5 to 15 min at 5°C . Incubation time for each of the mitochondrial enzymes

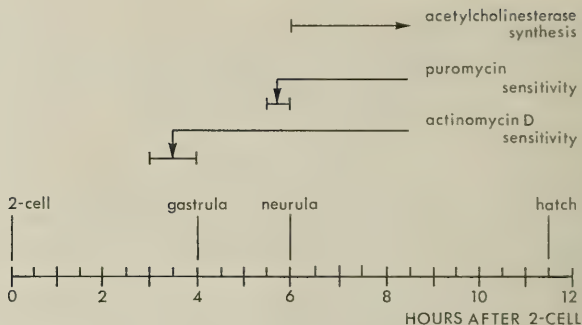


FIGURE 1. Time of various developmental stages and other events in specimens of *Molgula arenata* at 18° C.

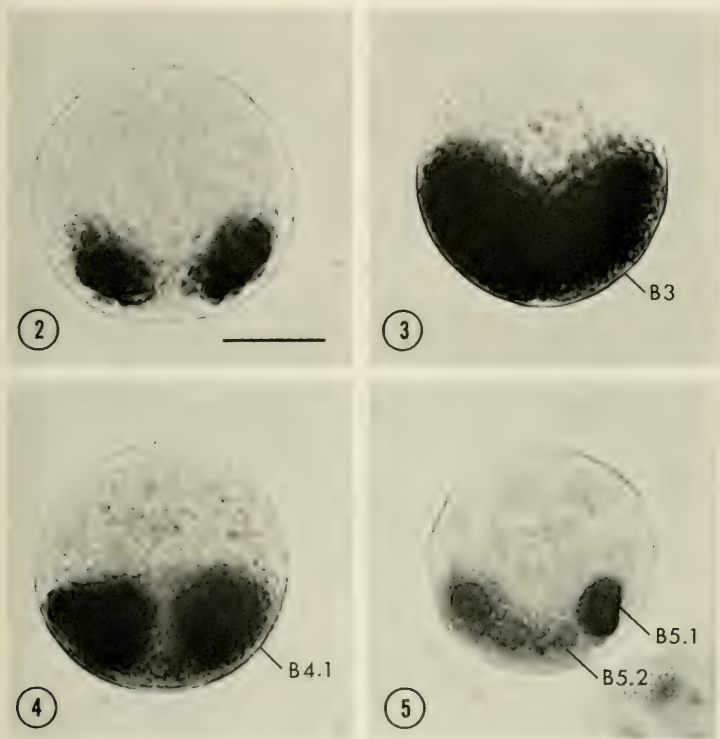
was 90 to 120 min at 22° C. Succinic dehydrogenase staining did not occur when sodium succinate was omitted from the incubation medium; the enzyme activity was not affected significantly by 10 mM sodium azide. Cytochrome oxidase activity did not occur in the presence of either 10 mM sodium azide or 10 mM potassium cyanide. Cytochrome c greatly enhances the sensitivity of the reaction but cytochrome oxidase gives a modest reaction with 3,3'-diaminobenzidine in the absence of substrate. Concentrations of the reaction products in both of these histochemical reactions appear to follow the Beer-Lambert law; color density is proportional to enzyme activity and therefore proportional to the numbers of mitochondria (Cabrini, Vinuales, and Itoiz, 1969; Marinos, 1978).

After the respective histochemical reactions the embryos were dehydrated in ethanol, cleared in xylene, and mounted in damar resin. The three enzyme procedures produced essentially permanent color reactions.

RESULTS

Development of acetylcholinesterase

Development of larval tail muscle acetylcholinesterase in *Molgula arenata* initially followed the same pattern found in urodele ascidian species: the enzyme became histochemically detectable at neurulation. Color first appeared at 6 hr after the 2-cell stage (Fig. 1) and enzyme activity accumulated gradually over the next few hours until a modest level of activity was attained (Fig. 2). In some larvae this activity reached a level as high as 20% of that found in the larvae of comparable urodele species of *Molgula*, but the mean activity found was 5 to 6% of the urodele level (Whittaker, 1979). Tail development was completely suppressed in *M. arenata* and except for acetylcholinesterase development presumptive muscle tissues did not otherwise develop beyond the early neurula stage; there was no obvious myofibrillar synthesis.



FIGURES 2-5. Embryos of *Molgula arcuata* stained histochemically for acetylcholinesterase.

FIGURE 2. Hatched larva.

FIGURE 3. Embryo cleavage-arrested in cytochalasin B at the 4-cell stage and reacted for enzyme at 8 hr after the time of the 2-cell stage.

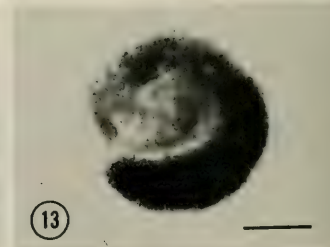
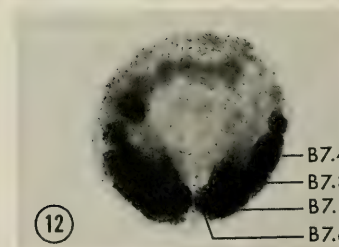
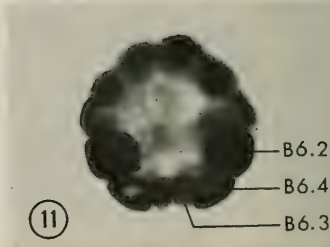
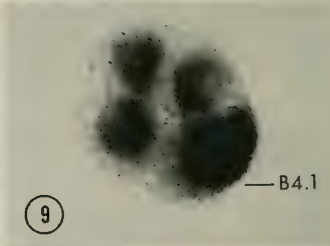
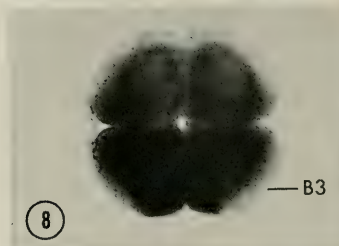
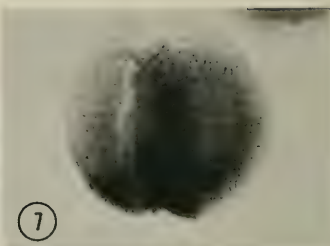
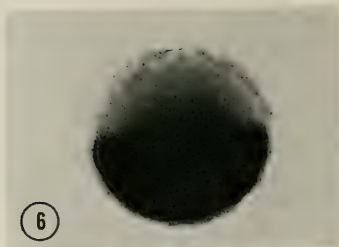
FIGURE 4. Embryo cleavage-arrested at the 8-cell stage and reacted for enzyme at 8 hr after the 2-cell stage.

FIGURE 5. Embryo cleavage-arrested at the 16-cell stage and reacted for enzyme at 8 hr after the 2-cell stage.

The muscle cell lineage designations are according to Ortolani (1955). All figures are the same magnification; the bar in Figure 2 is 40 μ m long.

Segregation of a muscle determinant

Previous studies with cleavage-arrested embryos of *C. intestinalis* have shown that blastomeres of the muscle lineage in arrested embryos will eventually develop acetylcholinesterase (Whittaker, 1973). Similar experiments with *M. arcuata*



produced the same results, at least in the earlier stages. Such observations support the theory that an autonomously acting cytoplasmic determinant of acetylcholinesterase development is being segregated in the muscle lineage cells.

Embryos of *M. arenata* were placed in 2 $\mu\text{g}/\text{ml}$ cytochalasin B (Sigma) at various cleavage stages after fertilization. These were then reacted for acetylcholinesterase 8 to 9 hr after the 2-cell stage. The maximum cell numbers producing acetylcholinesterase and the relative positions of these cells in the embryo matched those of the known ascidian muscle cell lineage: one blastomere at 1-cell, two at 2-cell, two at 4-cell, two at 8-cell, and four at 16-cell. Figures 3-5 depict the last three of these stages.

A majority of the embryos at all of the various cleavage-arrested stages developed acetylcholinesterase in one or more of the muscle lineage blastomeres and a modest number of embryos at 2-, 4- and 8-cell stages produced the maximum lineage numbers of reacting cells (two). Interestingly, few embryos at the cleavage-arrested 16-cell stage produced enzyme in more than two cells, one on each bilateral side. Figure 5 illustrates one of the embryos in which all four of the muscle lineage cells produced acetylcholinesterase. Usually only the B5.1 cells synthesize acetylcholinesterase. This restriction to two rather than four cells is conceivably related to the lesion that results in a limited expression of acetylcholinesterase in *M. arenata*.

A limitation of expression seemed also to occur in later stages as well, but there the results were less certain. The chorion of *M. arenata* adheres closely to the embryo and appears to exert tension during development. Consequently, the acetylcholinesterase-producing muscle lineage cells in cleavage-arrested 32-cell and 64-cell stages tend to remain aggregated together in two bilateral groups. Aggregation combined with high yolk content of the cells and the large number of nuclei which accumulate make it essentially impossible to discern cell boundaries within groups of older myoblasts.

Acetylcholinesterase dependence on protein and RNA synthesis

The time at which enzyme was first detected histochemically (Fig. 1) is apparently the time of first acetylcholinesterase synthesis. When 200 $\mu\text{g}/\text{ml}$ puromycin di-HCl (Sigma) was added to embryos of *M. arenata* 30 min before the time (at 6 hr) of first acetylcholinesterase staining no enzyme was detected histochemically at 8 to 9 hr time. Similarly, embryos placed in puromycin at

FIGURES 6-13. Embryos of *Ciona intestinalis* stained histochemically for succinic dehydrogenase.

FIGURE 6. Unfertilized egg.

FIGURE 7. 2-cell stage.

FIGURE 8. 4-cell stage.

FIGURE 9. 8-cell stage. Side view of the bilaterally symmetrical embryo.

FIGURE 10. 16-cell stage.

FIGURE 11. 32-cell stage.

FIGURE 12. 64-cell stage.

FIGURE 13. Middle tailbud stage at about the time of first melanocyte differentiation (12 hr development at 18° C).

The muscle lineage designations are according to Ortolani (1955). All figures are the same magnification; the bar in Figure 13 is 40 μm long.

6 hr produced, even after many hours, only the slight amount of enzyme activity that would ordinarily be detected at 6 hr time. Puromycin at 200 $\mu\text{g}/\text{ml}$ causes 95 to 99% inhibition of protein synthesis in ascidian embryos (Whittaker, 1973, 1977). Presumably the cytoplasmic factor being segregated is not a preformed inactive acetylcholinesterase since the occurrence of activity seems to depend directly on protein synthesis.

There was also an actinomycin D sensitivity period for acetylcholinesterase synthesis in this species. This occurred between 2 and 3 hr before the time of enzyme synthesis (Fig. 1). When embryos were treated continuously with 20 $\mu\text{g}/\text{ml}$ actinomycin D (Sigma, Grade III) beginning at 3 hr after the 2-cell stage, no acetylcholinesterase developed subsequently. If treatment was started at 4 hr some slight amount of enzyme activity was found at hatching time. Progressively more enzyme activity was found the later after 4 hr that actinomycin D treatment was started. Since actinomycin D at this concentration produces a maximal inhibition of RNA synthesis in ascidian embryos (Smith, 1967; Mansueto-Bonaccorso, 1971), occurrence of acetylcholinesterase probably requires a specific embryonic period of RNA synthesis. Enzyme messenger RNA (mRNA) synthesis most likely occurs during this time. On the basis of other studies (Whittaker, 1977), one can assume that enzyme synthesis would be resistant to actinomycin D if a performed mRNA for the enzyme were present.

Mitochondrial segregation in urodele embryos

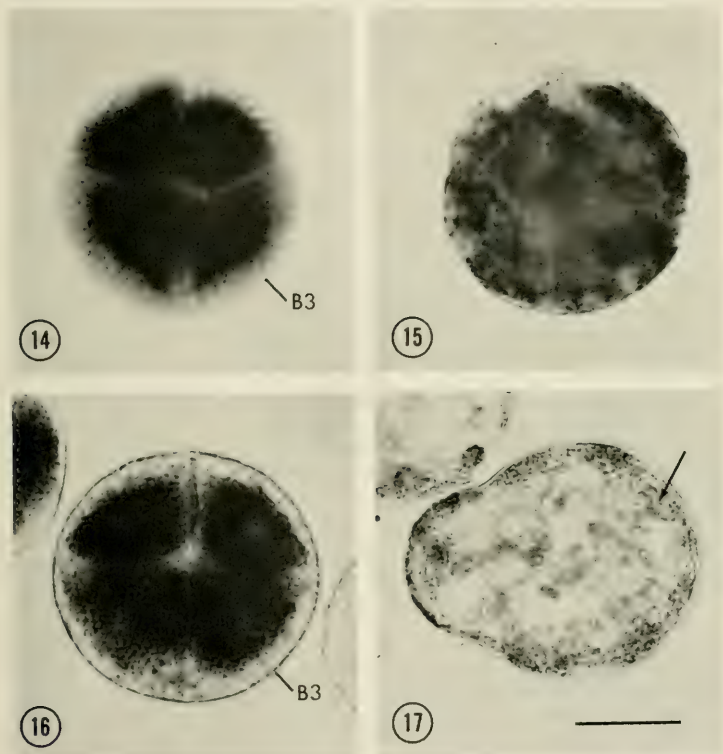
Succinic dehydrogenase reactions in early embryonic stages of *C. intestinalis* showed clearly the pattern of mitochondrial distribution in the embryos (Figs. 6-13). Before germinal vesicle breakdown, which occurs in the oviduct, mitochondria are distributed uniformly in the subcortical cytoplasm of the egg, as seen by the distribution of succinic dehydrogenase in cryostat sections (Patricolo, 1964). After germinal vesicle breakdown and before fertilization there is a migration of mitochondria to the vegetal half of the egg (Fig. 6). After fertilization they migrate further into the vegetal half of the egg and many of them eventually form a mitochondrial crescent, which is seen most clearly at the 2-cell stage (Fig. 7).

Although mitochondria were distributed elsewhere in the egg and embryos, a major concentration occurred in the muscle lineage cells, according to the lineage patterns established by Conklin (1905) and Ortolani (1955). One should note particularly the distribution of activity at the 4-cell stage (Fig. 8) where there is obviously segregation of much more activity into the B3 pair of blastomeres. At the tailbud stages one sees the strong localization of enzyme in the differentiating muscle cells of the tail (Fig. 13). Identical results were obtained with embryos of another species, *M. occidentalis*.

Similar enzyme distributions can be shown with a second mitochondrial enzyme, cytochrome oxidase. Histochemical localizations of this enzyme were identical in both *C. intestinalis* and *M. occidentalis* to those shown in Figs. 6-13 for succinic dehydrogenase. This duplication lends additional confidence to the likelihood that these staining reactions reveal the mitochondrial distribution rather than simply reactive peculiarities of a particular enzyme.

Mitochondrial enzymes in anural embryos

There was no evidence at any embryonic stage that mitochondria were differentially segregated into muscle lineage blastomers of *M. arcnata*. Embryos were examined at cleavage stages and at various later developmental stages. At second cleavage the two posterior (B3) blastomeres are slightly larger than the



FIGURES 14-17. Embryos of *Molgula arcnata* stained histochemically for the mitochondrial enzymes succinic dehydrogenase and cytochrome oxidase.

FIGURE 14. 4-cell stage embryo stained for succinic dehydrogenase.

FIGURE 15. Embryo 6 hr after the 2-cell stage stained for succinic dehydrogenase.

FIGURE 16. 4-cell stage embryo stained for cytochrome oxidase.

FIGURE 17. Hatching larva stained for cytochrome oxidase. The arrow indicates the position of one arm of the muscle cell crescent.

All figures are the same magnification; the bar in Figure 17 is 40 μ m long.

anterior pair. Figure 14 shows that distribution of succinic dehydrogenase occurs in proportion to the size of the blastomeres. There is no obvious differential segregation of activity into the posterior pair. At the neurula stage (Fig. 15) the developing larva had no enzyme localization in those presumptive muscle rudiments that so clearly showed the presence of acetylcholinesterase in other experiments (Fig. 2). Cytochrome oxidase staining revealed the same result: no segregation of enzyme at either the 4-cell stage (Fig. 16) or in the hatching larva (Fig. 17). As described in detail elsewhere (Whittaker, 1979), the hatching larva emerges "head" end first from the chorion; in Fig. 17 the smaller bulge at the left is the emerging anterior end of the larva. The crescent of myoblasts is located at the position of the arrow. There is no differential accumulation of cytochrome oxidase staining in this region of the embryo, or elsewhere.

DISCUSSION

Preformation of mitochondria in the ascidian oocyte is an apparent adaptation to the needs of rapid development in oviparous embryos. Presumably the embryo is unable to synthesize sufficient mitochondria during a brief development time to meet the high energy requirements of larval tail muscle, and must prepare some of these in advance of embryonic development. Ascidian embryos synthesize additional mitochondria during development. Mancuso (1962) noted bilobed mitochondrial structures especially in myoplasmic regions of the *Ciona* embryo, and concluded that muscle mitochondria multiply during embryogenesis. Measurements of mitochondrial enzyme activity indicate that only about half of the number of mitochondria found at larval hatching occur preformed in the fertilized egg (D'Anna and Metafora, 1965; D'Anna, 1966).

According to enzyme activity measurements by Berg (1956, 1957) on separated blastomeres at the 4-cell stage roughly two-thirds of the original mitochondria are segregated (in *Ciona*) into the two B3 muscle lineage blastomeres. A large proportion of these become actively segregated into the later cells of the muscle lineage. While mitochondrial preformation and segregation may be necessary for optimal physiological function of the larva at hatching, the important question is whether this initial concentration directly provides information in the developing muscle system.

Larvae of most ascidian species have a histospecific acetylcholinesterase that occurs in the tail muscle cells (Durante, 1956, 1959). One anural species, *M. arcuata*, also developed enzyme in the presumptive muscle cells of the aborted tail (Whittaker, 1979), which indicates that these cells are unquestionably programmed to become muscle. Although myofibrils were not obvious, and probably did not occur, the cells nevertheless differentiated part of their histospecific acetylcholinesterase.

Evidence from experiments with cleavage-arrested embryos of *Ciona* (Whittaker, 1973) and microsurgically isolated partial embryos (Whittaker, Ortolani, and Farinella-Ferruzza, 1977) suggests that ascidian embryos localize and segregate a cytoplasmic determinant concerned with synthesis of acetylcholinesterase by the developing muscle cells. This factor appears to be neither the enzyme itself nor a preformed mRNA for acetylcholinesterase, and is probably an agent responsible

for activating particular genetic programs in the embryo (Whittaker, 1973). A similar determinant apparently occurs in *M. arcnata*. Segregation of a determinant was demonstrated by cleavage-arresting embryos of *M. arcnata* with cytochalasin B, and finding the eventual development of acetylcholinesterase in blastomeres of the ascidian muscle lineage pattern. Also, normal occurrence of acetylcholinesterase activity was blocked by puromycin and actinomycin D, suggesting thereby that there is neither preformed enzyme nor preformed mRNA for acetylcholinesterase.

Histochemical staining of the mitochondrial enzymes succinic dehydrogenase and cytochrome oxidase in embryos and larvae of *M. arcnata* showed unequivocally that no "crescent" localization of mitochondria occurred after fertilization and no subsequent differential segregation of mitochondria took place. The conclusion that anural embryos lack a differential localization and segregation of mitochondria is thought to be valid for two reasons. Results with the same techniques in a normal urodele embryo series (Figs. 6-13) unambiguously show differential mitochondrial distribution. Secondly, concentrations of the histochemical reaction products follow the Beer-Lambert law, and the human eye is sufficiently sensitive to distinguish even minor concentration differences in the products.

In *M. arcnata* there is an uncoupling of mitochondrial behavior and muscle cell differentiation. Mitochondria are not localized and not differentially segregated; yet there is determination of larval muscle and its partial differentiation. This shows without question that the mitochondrial accumulation in muscle cells of ascidian embryos has no direct informational relationship to the cellular differentiation. At the same time it is likely that the cytoplasmic determinants of muscle are not physically associated with the myoplasmic mitochondria. One cannot, however, rule out the possibility that the same physical mechanism is involved in both mitochondrial segregation and segregation of the determinants responsible for muscle differentiation. If so, the processes operate independently of one another.

The demonstration of disjunction between mitochondrial segregation and muscle differentiation is a potent example of the intrinsic superiority of nature's experiment. Theodore Boveri urged us long ago to seek the natural experiment where possible: "the investigator of living processes will make it his special concern to find out abnormalities, in which he has not intervened with his crude methods, where he can penetrate into the nature of the alteration" (Boveri, 1908, p. 216).

I thank Dr. Arthur Humes, Director of the Boston University Marine Program at the Marine Biological Laboratory, for the use of dredging facilities, and Mr. Charles H. Henry Jr. of the Case Western Reserve University Dental School for his patient preliminary study of succinic dehydrogenase staining in embryos of *Ciona*. The work was supported by Grant HD 09201, awarded by the National Institute of Child Health and Human Development, DHEW.

SUMMARY

The ascidian *Molgula arcnata* produces an anural larva in which development of the tail and other urodele features has been suppressed. The embryos never-

theless developed part of the histospecific tail muscle acetylcholinesterase; the presumptive myoblasts have obviously acquired the muscle differentiation program. When cleavage-stage embryos were prevented from undergoing further division by treatment with cytochalasin B, acetylcholinesterase eventually developed in blastomeres of the muscle lineage. These anural embryos apparently segregate a cytoplasmic determinant concerned with acetylcholinesterase development into cells of the muscle lineage. In this species there was no localization and segregation of mitochondrial succinic dehydrogenase and cytochrome oxidase in the muscle lineage, as found in embryos of two urodele ascidians, *Ciona intestinalis* and *Molgula occidentalis*. The causal determinant of histospecific acetylcholinesterase expression is not, therefore, a differential localization of mitochondria nor is segregation of the muscle determinants linked directly to mitochondrial segregation.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

Abstracts are arranged alphabetically by first author. Author and subject references will be found in the regular volume index, appearing in the December issue.

GENERAL SCIENTIFIC MEETINGS

AUGUST 20-22, 1979

Morphogenesis in grafted Hydra attenuata: positive dominance, negative dominance, and pattern regulation. JAMES ALFRED ADAMS.

Positive dominance (induction) and negative dominance (inhibition) have been studied in grafted *Hydra attenuata*. Grafted animals are prepared having three gastric regions, tandemly arranged, with original disto-proximal orientation of the graft pieces being maintained. Grafted animals prepared in this fashion (3g hydras) have the ability to regenerate heads and feet in the vicinities of the graft borders. These regenerates are referred to as a secondary head (2^h) and secondary feet (2^f). Gradients in the frequencies of both 2^h and 2^f regeneration are observed in these animals. Using Chi-square methods and a 95% level of confidence, one finds a significantly greater frequency of 2^h regeneration at the graft border farthest from the terminal head ($0.050 > P > 0.010$). Likewise, a higher frequency of 2^f regeneration occurs at the graft border farthest from the terminal foot ($0.005 > P$). These data suggest that the terminal head or foot, by its presence, either inhibits or causes inhibition of a like structure occurring (negative dominance). This inhibitory effect decreases in a linear fashion with increasing distance from the terminal head or foot as the case may be.

However, when a tentacle and a small piece of attached hypostomal tissue is left on the middle and proximal graft pieces there is no inhibition of head formation at either graft border. This result shows that the inductive capacity (positive dominance) of the tentacle-hypostome region can overcome the negative dominance of the terminal head. This technique also affects 2^f formation on the 3g animal. The frequency of 2^f regeneration is enhanced significantly at the proximal graft border and decreased significantly at the distal border, with the effect that the gradient in foot formation observed in control 3g animals is abolished. Thus, a direct relationship, and perhaps an interdependency between the gradients of 2^h and 2^f regeneration in 3g *H. attenuata* is demonstrated.

Alternate pathways in the biosynthesis of testosterone by Rana pipiens.
CHRISTOPHER A. ADEJUWON, SHELDON J. SEGAL, AND S. S. KOIDE.

The absence of immunoreactive progesterone, despite the presence of appreciable amounts of immunoreactive testosterone in unstimulated testes taken from specimens of *Rana pipiens*, led us to investigate the precursor role of progesterone in testicular testosterone biosynthesis in this amphibian species. Three sets of experiments were performed. The first was chromatographic analysis of testicular steroids. Testis slices (60-80 mg) were placed in 2.0 ml ringer's solution and were agitated with 10.0 ml petroleum ether and then with chloroform. After evaporating the solvent, the residue was dissolved in chloroform:methanol and applied to fluorescent-coated silica gel thin-layer chromatography (TLC) plates. The chromatogram was developed in ethyl acetate:water:n-hexane:ethanol. The resulting spot was visualized with ultraviolet light using testosterone as a marker. The steroid present in the testicular extract had an R_f value of 0.63 compared to the R_f values of 0.67 and 0.72 for testosterone and progesterone, respectively. Radiolabeled pregnenolone co-migrated with progesterone in this system.

In the second experiment, *in vitro* stimulation of testosterone production by human chorionic gonadotropin (hCG) was demonstrated by incubating *Rana* testis slices with ringer's solution containing varying concentrations of hCG. Aliquots (100 μ l) of the incubation medium were removed between 0.5 and 2.0 hr of incubation for testosterone determination

by radioimmunoassay (RIA). Testosterone production was stimulated by hCG in a dose-related manner; the lower limit of sensitivity was 2 ng. Thirdly, *in vitro* conversion of precursor to ^3H -testosterone was demonstrated by incubations of ^3H -progesterone and ^3H -pregnenolone with testicular slices in Ringer's solution containing a nicotinamide adenine dinucleotide phosphate-generating system. Steroids were extracted and analyzed on TLC. About 8.8% of ^3H -progesterone and 8.7% of ^3H -pregnenolone were transformed to ^3H -testosterone in 4 hr of incubation. The present results, based on RIA, TLC, and hCG-stimulation show that testosterone is produced by testes taken from mature male frogs of the *R. pipiens* species. The Δ^5 and Δ^4 biosynthetic pathways appear to be equally important for *in vitro* testosterone formation. HCG concentrations as low as 2 ng stimulate testosterone production.

Supported by a grant from the George Hecht Fund. C. A. Adejumo is a recipient of a Rockefeller Foundation Fellowship.

Characterization of Spisula sperm protamine. JUAN AUSIO AND K. E. VAN HOLDE.

The major protein component of mature *Spisula* sperm nuclei appears to be a protamine. This material has been purified from ripe sperm by the following procedure: sperm were washed gently from excised gonads and homogenized in 5% acetic acid. After low-speed centrifugation, the nuclei were re-extracted with 25% acetic acid. These extractions remove most of the contaminating histones. The nuclei were next extracted with 0.25 N HCl, and the protamine so released was purified by chromatography on a CMC-25 column, using 1.2–1.8 M NaCl gradients.

The protamine so prepared migrated as a single band on urea gel electrophoresis, with the reservation that a small amount of material always remained at the top of the gel. Sedimentation equilibrium studies at pH 9.2, and treatment with 0.4 M NaCl revealed a nearly homogeneous material of molecular weight 29,400. The sedimentation coefficient was determined at a number of concentrations in 0.4 M NaCl, and the extrapolated value of $S_{20,w} = 2.0 \times 10^{-13}$ was obtained. This value, small for a protein of this mass, corresponds to a frictional ratio $f/f_0 = 1.55$, indicating asymmetry or unfolding of the molecule. Upon increasing the ionic strength above 1 M, the sedimentation coefficient increases about 25%, probably from a condensation of this highly charged molecule.

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Phosphorylation of the 40s ribosomal subunit after fertilization of Arbacia punctulata eggs. DENNIS BALLINGER, SCOTT PETERSON, AND TIM HUNT.

A particularly prominent alteration in the pattern of protein phosphorylation after fertilization of *Arbacia punctulata* eggs occurs in a 31,000 MW protein whose labeling increases relative to other proteins. This increase can be detected 5 min after fertilization in eggs which have been labeled for 2 hr with $^{32}\text{P}_i$.

The following criteria identify this protein as a component of the 40s ribosomal subunit: first, it co-sediments with 80s ribosomes on a low salt sucrose gradient; secondly, it co-sediments with the 40s ribosomal subunit on a high salt sucrose gradient; thirdly, its mobility in a two dimensional gel system designed to separate basic ribosomal proteins (acid urea/SDS) suggests that it may be the sea urchin analogue of protein S6 of yeast and mammalian ribosomes, but this identification is tentative at present. We hope to use this analytical system to determine whether the increased labeling represents *de novo* phosphorylation, or simply an increased rate of turnover of preexisting phosphate residues. Our data indicate, however, that at least one of these parameters must change after fertilization.

We have made one test of the hypothesis that the phosphorylation of this ribosomal protein may be connected in some way with the 20- to 30-fold increase in protein synthesis which occurs in the sea urchin after fertilization by asking whether the labeled subunits were preferentially associated with polysomes. We labeled embryos with $^{32}\text{P}_i$ for 4 hr following fertilization, and analyzed extracts on sucrose gradients. The labeled 31,000 MW protein was found associated to the same extent with 80s ribosomes and polysomes in terms of cpm/ A_{260} . These results are

indicative of little or no preferential association of phosphorylated subunits with mRNA in 4-hr embryos. Thus, the significance of this phosphorylation, while suggestive, remains uncertain.

Difference between SDS-PAGE patterns of Labyrinthula slimeways and vegetative cells. EUGENE BELL, NORIO NAKATSUJI, AND STEPHANIE SIHER.

The slimeways of *Labyrinthula* are synthesized by the cells and contain contractile proteins organized, in part, into filaments. To examine the relative protein complexities of cells and slimeways, colonies were labeled with ^{14}C reconstituted protein hydrolysate, homogenized, fractionated into cells and slimeways and then subjected to SDS PAGE. Labeled homogenates of whole colonies gave a minimum of 55 radioactive bands. Bands comigrating with chick skeletal muscle myosin and chicken gizzard were identified. The slimeway fraction showed five times fewer bands than the whole colony homogenate or the cell fraction homogenate, but the film densities of actin bands of the cell and slimeway fractions was the same, while the myosin band of the slimeway fraction was even more dense.

To try to understand the segregation of cell and slimeway proteins in the three-dimensional colony, we have reviewed the fine structure of *Labyrinthula* and propose the following scheme: In the extrusion of "extracellular" membranes and slimeway proteins from a cell bounded only by its plasma membrane (cell membrane I), the first event important for slimeway formation may be the ballooning of membrane vesicles from bothrosome apertures. The vesicles with associated proteins flatten into two layers and open where the layers make hairpin turns. The layer adjacent to the cell, cell membrane II, which is attached to the cell through the bothrosome, fuses with comparable layers from other bothrosomes of the same cell to form a second membrane around the cell. The cell is now invested with a second membrane of reverse polarity with respect to the plasma membrane. Thus, through vesicle formation and rupture, selected proteins and formed membrane pieces are brought to the exterior for slimeway construction.

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Conduction and dielectric studies of protein-methylglyoxal complexes. STEPHEN BONE.

Dielectric and conduction studies of bovine serum albumin (BSA), casein, collagen, and lysozyme treated with methylglyoxal have been made in an attempt to provide evidence for the existence of the charge-transfer mechanism proposed by Albert Szent-Györgyi (1979, *Biol. Bull.*, **157**: 398). The dry treated protein samples show a dielectric dispersion at sub-Hertz frequencies whereas no such trend is observed for the pure proteins. A similar low frequency dispersion has been shown to exist in the dielectric studies of the conventional charge-transfer complex (perylene + chloranil). These dispersions have been found to be associated with a bulk rather than an electrode interfacial effect. The observation of an increased dc conductivity and lowered conductivity activation energy for the protein-methylglyoxal complexes as compared with the untreated proteins is consistent with the formation of a charge-transfer complex. Analysis of the dielectric dispersions indicate that this dielectric loss cannot be interpreted using conventional dipolar relaxation theories, but they may be understood in terms of the hopping of mobile charge carriers. These carriers are thought to hop over potential energy barriers associated with intra- and inter-protein boundaries, and their mean free path length has been determined to be of the order of 1.5-1.8 nm for the BSA, casein, and lysozyme complexes and 16 nm for the collagen complex. These studies provide strong evidence for the existence of a charge-transfer interaction between proteins and methylglyoxal as originally proposed by Szent-Györgyi.

Pressure-flow relations in the perfused systemic circulation of squid. GEORGE B. BOURNE.

An artificially perfused preparation of the posterior systemic circulation of squid was developed to permit examination of some physical and cholinergic factors affecting pressure-

flow relations. The posterior aorta was cannulated via the systemic heart, after which pressure was monitored by a strain-gauge manometer connected to the cannula at a T-junction by polyethylene tubing. Flow was measured by an electromagnetic flowmeter and also was monitored by sensing loss of fluid from the perfusion reservoir via an isometric muscle lever. The signals from the transducers were read out on a Brush recorder.

The preparation took approximately 15 min to stabilize when perfused by vigorously oxygenated, filtered sea water. Stability was maintained for 1 to 2 hr, after which the preparation decayed rapidly. The decay was characterized by increasing vascular resistance and severe oedema.

When flow was plotted against pressure, a curve which obeyed a power function was obtained. Acetylcholine caused a marked vasodilation. The threshold of response to acetylcholine was 10^{-11} M with 75% of maximal response occurring at 10^{-8} M. The vasodilatory action of acetylcholine was blocked by *d*-tubocurarine.

This work was supported by a Steps Toward Independence Program Fellowship and by an operating grant from the National Scientific and Engineering Research Council of Canada.

The numbers and sizes of cells in molluscan ganglia: simultaneous optical recording of activity from many neurons. [Demonstration.] M. B. BOYLE, L. B. COHEN, AND E. R. MACAGNO.

We have compared ganglia from the central nervous systems of a number of gastropod molluscs with the object of finding an animal having relatively large and relatively few nerve cells. We hope to use optical methods for monitoring activity to help us unravel the neuronal basis of behaviors. Freshly dissected ganglia were left in a 0.1 to 0.3 mg/ml solution of methylene blue in saline for 12 to 18 hr at room temperature, fixed in 4% formaldehyde in saline for about 30 min, dehydrated in 30, 60, 90, and 100% acetone, and cleared in methyl salicylate. The ganglia were then whole-mounted between cover slips using Permount. This procedure is designed to selectively stain cell bodies. We showed examples of buccal ganglia from *Aplysia californica*, *Dendronotus iris*, and *Tritonia diomedea*, and of circumesophageal ganglia from *Hermisenda crassicornis*, *Dendronotus iris*, and *Tritonia diomedea*. As viewed under the dissecting scope, the buccal ganglia of *Tritonia* and *Aplysia* appear to contain a relatively large number of small cells as compared with *Dendronotus*. The circumesophageal ganglia of *Hermisenda* seem to consist of cells whose average size is significantly smaller than that in either *Tritonia* or *Dendronotus*. *Dendronotus* appears to have relatively few small cells in either the buccal or the circumesophageal ganglia.

We also showed an example of a pre-recorded experiment done at Friday Harbor Laboratories on *Dendronotus iris*. We detected spontaneous activity and activity in response to suction electrode stimulation of nerves from a circumesophageal ganglion using the merocyanine-rhodanine dye WW433.

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Optimal prey selection of Littorina littorea by green crabs. JOHN E. BOYNTON.

Optimum foraging theory predicts that predators will be adapted to select prey of a size that returns maximal food energy for time invested. *Carcinus maenas* (green crab) has a varied diet which includes *Littorina littorea* (periwinkle). Crabs attack snails by several methods: crushing, shell edge chipping, and shell boring. Predictions of optimal foraging theory and correlations of attack armature and defensive armor were tested using this predator-prey system. Snails of all sizes were fed to a size-spectrum of individual crabs, and prey size selected was determined. Snail shell-length: dry-weight relations were measured. Parameters of crab claws were examined as well as snail shell thickness and length relations. Only snails in the size range of 5 to 17 mm were selected by crabs. Dry weight results indicate that snail soft tissue increases exponentially. Crabs would benefit more from going after larger snails rather than smaller snails. Crabs mostly crushed their prey. Crabs chose snails that fell within the upper range of that permitted by the individuals' claw gape, and

ignored smaller snails. This corresponds with data on snail populations in the salt marsh. Claw size seems to determine the prey size that can be attacked.

Chemoreception in catfish: effects of diet on behavior and body odor. BRUCE BRYANT AND JELLE ATEMA.

Whole body-odor complexes are important in maintaining social structure, but this maintenance is not based on a rigid stimulus-response relationship as found in typical pheromone-mediated social behavior. In aquaria, the brown bullhead, *Ictalurus nebulosus*, forms territories and dominance hierarchies. The importance of body odor in maintaining these territories and hierarchies was investigated by observing the effects of a changing body odor of one member of a territorial pair. Body-odor change was induced by a behaviorally non-invasive manipulation—a change in diet.

Pairs of fish, fed identically for at least three weeks (Purina trout chow), were observed and both social and solitary behavioral units were recorded. A handling control preceded the diet manipulation and consisted of isolating and feeding the subdominant (β) fish on chow in a separate tank for 1 day. Two days after reintroduction into the main tank, (β) was isolated again but this time fed an identical volume of beef liver. In three of four tanks, a change from display behavior to aggressive nips and bites by α occurred when comparing control and experimental reintroductions of β . The fourth tank showed no change in aggressive behavior but a change in territorial status. β 's territory suffered significantly more incursions following manipulation than during the control periods ($P < 0.05$, Friedman 2-way ANOVA). Inspection of β 's solitary behavior units did not reveal effects of the diet change. The changes in behavior associated with a change in diet suggest that the maintenance of territorial hierarchy is dependent on chemical recognition of conspecifics which have become familiar; diet manipulation disrupts this recognition. The degree and time course of this disruption seems to suggest that it is not complete and that familiarity is regained within days.

New developments in the mariculture of Aplysia californica. THOMAS R. CAPO, SUSAN E. PERRITT, AND CARL J. BERG, JR.

It recently has become possible to study the developmental neurobiology of *Aplysia californica* through the use of laboratory cultured animals. In order to optimize culture conditions we have emphasized three approaches. The first involved the evaluation of various antibiotics. The combination of Penicillin-G sodium salt (100.5 units/ml) and Streptomycin-sulfate (50 μ g/ml) was routinely found to improve the survival of veliger cultures. The second approach was to find substrates other than *Laurencia pacifica*, for triggering the metamorphosis of *A. californica*. Two red alga available in the Woods Hole region, *Neogardheilla baileyi* and *Gracilaria* sp., have been successful. *Neogardheilla baileyi*, Strain A₁, has been cultured in the laboratory for six months (T. Capo, in preparation). With present techniques, more than adequate amounts of epiphyte-free *N. baileyi* have been grown for metamorphic and early juvenile stages. Finally, the technique for handling metamorphic stages has been simplified with the use of disposable 50-ml conical centrifuge tubes. By using sufficient algae to cover the base of the tube, sinking animals are brought into increased contact with the substrate on which metamorphosis takes place. The combined effects of these three approaches have simplified and improved the mass culture of *Aplysia californica*.

This work was funded by a grant from the Klingenstein Foundation.

Motor fields of pharyngeal motoneurons in Navanax, an opisthobranch mollusc. MITCHELL S. CAPELL, DAVID C. SPRAY, DAVID H. HALL, ABRAHAM J. SUSSWEIN, AND MICHAEL V. L. BENNETT.

Three orthogonal muscle groups comprise the *Navanax* pharynx: radials which run through the pharyngeal thickness and mediate expansion, and superficial circumferentials and longi-

tudinals which cause constriction and shortening. Stimulation of identified neurons in the buccal ganglion cause these pharyngeal movements. This work provides evidence that these neurons are true motoneurons, with specific and reproducible motor fields. Evidence indicating that these cells directly innervate muscle includes: constant latency EMGs are evoked by intrasomatic stimulation at moderately high frequencies, antidromic impulses are evoked by electrically stimulating the appropriate muscle area, and a peripheral nerve net is apparently absent.

Circumferential and longitudinal muscles consist of a superficial gridwork of a very consistent number of discrete bands. Localizability and reproducibility of motor fields was quantitatively determined with respect to these pharyngeal landmarks.

Motoneuron fields were identified by: movements following intrasomatic stimulation, area producing 1-1 EMGs with intrasomatic impulses, areas yielding antidromic impulses at low threshold, and axonal course through nerves innervating specific areas. Generally, these methods gave mutually consistent results.

To date fields of 10 motoneurons have been characterized. These fields show little variability. Four circumferential motoneurons, two per side, innervate fused circumferential bands located at the anterior of the pharynx. Of the two per side, one innervates ipsilaterally, the other bilaterally. Another left circumferential motoneuron bilaterally innervates the fused bands located posteriorly. Both giant (G) expansion motoneurons innervate radial muscles of the entire pharynx. The ventral left medium-sized (M) cell innervates the anterior half of the M cell. About another dozen observed fields are not yet adequately established. These include longitudinal motoneurons, none of which have been discovered previously. These data provide a further step towards understanding the neural control of feeding in *Navanax*.

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Ionophore induced sodium loading of nerve terminals: a model for long term facilitation of transmitter release. MILTON P. CHARLTON, CHARLIE S. THOMPSON, AND HAROLD L. ATWOOD.

Accumulation of sodium ions caused by sodium pump inhibitors, metabolic poisons, repetitive stimulation or direct injection increases spontaneous and evoked transmitter release at synapses. We induced sodium loading in nerve terminals by application of the sodium ionophore, Monensin. In the stretcher muscle of the spider crab (*Libinia*), application of 6 μ M Monensin caused a 2- to 8-fold increase in the amplitude of postsynaptic potentials (psp) evoked when the excitator motoneuron was stimulated. Recovery following washout of Monensin took up to 50 min. A second dose gave a smaller response or none. The rate of increase of psp's following application of Monensin was greater in saline containing the normal concentration of sodium than in saline in which two-thirds of the sodium had been replaced by sucrose. This is consistent with a sodium ionophore role for Monensin in this system. Following application of Monensin in saline containing no calcium, normal saline (no Monensin) was reapplied and there was an immediate increase in psp amplitude as if Monensin had been working during its application in zero calcium saline. This and its miniscule calcium transporting ability militate against a calcium ionophore role of Monensin in our experiments. Extracellular focal recordings at synaptic sites showed that quantal content was increased by Monensin. Spontaneous transmitter release was increased by Monensin in crab, lobster and frog (cutaneous pectoralis) muscles; in the latter there was a 20-fold increase in spontaneous release. No changes in membrane potential or input resistance of muscle fibers were seen with Monensin treatment. The amount and duration of long lasting potentiation produced by tetanic stimulation were similar to the effects produced by Monensin. The data support a role for sodium in long-term facilitation and demonstrate the usefulness of Monensin for loading sodium in small cells.

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Some biochemical properties of an RNA-instructed DNA polymerase in developing sea urchin (Lytechinus pictus) embryos. BEVERLY S. CHILTON, MARC R. LAUFER, AND SANTO V. NICOSIA.

The rate of protein synthesis in unfertilized (UF) sea urchin eggs increases dramatically during early cleavage. This has been attributed to increased oogenic mRNA availability and translational efficiency. The presence of an RNA-instructed DNA polymerase in UF eggs and early cleavage stage embryos suggests that the sea urchin may inherit a mechanism for the amplification of existing maternal mRNA's.

An RNA-instructed DNA polymerase was partially purified according to Slater and Slater (1972, *Nature New Biology* 237: 81-87) from UF eggs and early (2-16 cell) embryos. Using the synthetic template-primer (rA)_n-(dT)_n, incorporation of (³H)-dTTP (305 cpm/pM) by the polymerase from UF eggs and 16-cell embryos was maximal at 15° C. Incorporation kinetics were linear for 150 min. The effects of polymerase concentration on the rate of DNA synthesis resulted in similar sigmoidal curves for the enzyme from both UF eggs and 16-cell embryos. This suggests that the polymerase has a similar subunit structure and relationship to template-primer prior to and during embryogenesis.

Polymerase activity, expressed as pm (³H)-dTTP/μg protein, was 0.17 and 0.53 for UF eggs (n = 3 animals/determination), and averaged 0.64 for two- and four-cell embryos. Specific activity (mean ± s.e.) was not significantly different for UF eggs (0.45 ± 0.08) compared to 16-cell embryos (0.55 ± 0.14). Thus RNA-dependent DNA polymerase is detectable throughout early development. However, when expressed on a per cell basis, specific activity decreases during this time. The partially purified polymerase from 16-cell embryos banded at a sucrose buoyant density of 1.15 to 1.16 g/ml, the same density as the particulate polymerase isolated from human tumors. This implies a viral association for the sea urchin RNA-dependent DNA polymerase which may play a role in early development.

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Hydrogen peroxide release from sea urchin eggs during fertilization: importance in the block to polyspermy. MICHAEL COBURN, HERBERT SCHUEL, AND WALTER TROLL.

Polyspermy results in abnormal cleavage and halting of development by the blastula stage in sea urchins. For successful fertilization polyspermy must be avoided. We noted a novel block to polyspermy employed by *Arbacia punctulata*: the release of hydrogen peroxide after fertilization. We suggest that this hydrogen peroxide inactivated other sperm after entry of the first, ensuring proper development.

We have demonstrated the crucial role of hydrogen peroxide release in preventing polyspermy in *Arbacia* by causing 100% polyspermy through the addition of catalase to the sea water in which eggs were fertilized. Catalase is an enzyme of high specificity for the decomposition of H₂O₂ to water and oxygen. An early finding was confirmed by showing that sperm are inactivated by minute concentrations of H₂O₂, and that this action was blocked by catalase. We have further shown that catalase produces polyspermy within the first minute after fertilization, when present at the time of the addition of sperm to eggs.

Protease inhibitors such as soybean trypsin inhibitor (SBTI) have been shown to cause polyspermy in *Arbacia*. This and other protease inhibitors also act to prevent oxygen uptake and H₂O₂ production in human white blood cells. We now note that SBTI prevented the release of H₂O₂ from fertilized *Arbacia* eggs, similar to its action in white cells. Thus, the cause of polyspermy produced by protease inhibitors could be the inhibition of H₂O₂ release, rather than the prevention of normal raising of the protective fertilization envelope as was suggested previously. These observations reinforce the relationship between proteases and H₂O₂ production in biological systems.

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Association of centriole-like structures with the marginal band of a molluscan red cell. WILLIAM D. COHEN AND IRIS NEMHAUSER.

Marginal bands (MBs) are continuous circumferential bundles of microtubules found classically in all non-mammalian vertebrate erythrocytes and in platelets. MBs are believed to function in cellular morphogenesis, but relatively little is known about their formation.

We have been engaged in a continuing survey of blood cells of local (MBL) invertebrates for possible presence of MBs, with the objectives of establishing the phylogenetic distribution of the MB system identifying useful cell types for experimental work on MB structure and physiology. Previously we have established that MBs are present in sipunculan red cells, *Limulus* amoebocytes, and lobster and crab coelomocytes. We report here the presence of MBs in the hemoglobin-containing red cells of the arks *Anadara ovalis* and *Anadara transversa*. A striking feature of these molluscan red cell MBs is the obvious presence, on or adjacent to each MB, of a pair of centriole-like structures which we have tentatively named "perioles". The living red cells (*A. transversa*) are highly flattened and generally slightly elliptical (10–15 μ m long axis). In cells lysed with Triton X-100 under microtubule-stabilizing conditions, the nucleus, cytoplasmic particles, and MB with associated perioles are directly observable in phase contrast (oil immersion). The MBs are usually elliptical, with the periole pair appearing at various points on the ellipse but most frequently near one end. The two perioles are normally adjacent; however, in some cases a gap (0.25–1.5 μ m) occurs between them. In fixed, uranyl acetate-stained whole mounts of lysed cells (TEM) MB microtubules and the densely-staining perioles are readily visible. A trans-MB network of fine filaments is also present. The perioles appear to be within or on the MB, with extra-MB microtubules often 'focussing' upon them in pole-like fashion. Their centriole-like features as observed in whole mounts include (a) close pairing, sometimes with approximately right angle positioning, (b) cylindrical appearance with one closed end, and (c) size, approximately $0.25 \times 0.17 \mu$ m. We suggest that the perioles may be MB organizing centers, functionally analogous to centrioles and basal bodies.

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Dependence of discrete wave frequency on pH in Limulus ventral photoreceptors.
D. WESLEY CORSON AND ALAN FEIN.

Discrete waves in the ventral photoreceptors of *Limulus* are thought to result from either direct or spontaneous activation of visual pigment molecules by the absorption of photon energy or thermal kinetic energy. We have found that the average frequency of occurrence of spontaneous discrete waves in darkness rises 15-fold over a 2 pH unit range (6.0–8.0) of artificial sea water that bathes the receptors. Over this same range, the increment of discrete wave frequency added by a steady dim light remains essentially constant.

Artificial sea water was buffered with 10 mM each of Pipes (pK=6.8), Hepes (pK=7.55) and Tris (pK=8.30) and titrated to pH values in the range from 6.0 to 8.0 in half pH unit increments. The frequency of discrete waves was determined by counting the number of discrete waves in five 40-sec intervals at each experimental pH after a 10 min exposure. A dim steady light was adjusted to give an increment of approximately 1 discrete wave per second (0.99 ± 0.66 dw/sec; $\bar{x} \pm$ s.d.; $n=9$) over the spontaneous frequency (0.89 ± 0.42 dw/sec, $n=9$) at pH 7.0. Dim 20-msec test flashes showed that changes in the average discrete wave frequency were not the result of changes in the average discrete wave amplitude, and data were taken only from cells in which the spontaneous discrete wave frequency returned to its control level following each exposure.

In seven of nine cells, the spontaneous discrete wave frequency rose within the pH range of 6.0 (0.16 ± 0.17 dw/sec, $n=4$) to 8.0 (2.41 ± 0.60 dw/sec, $n=4$), while in the remaining two cells there was no appreciable rise in frequency from pH 7.0 (0.17 ± 0.06 dw/sec) to pH 8.0 (0.17 ± 0.10 dw/sec). No appreciable rise in the light-induced frequency increment occurred in either the seven responsive cells (1.30 ± 0.33 dw/sec at pH 6.0 vs. 0.79 ± 0.76 dw/sec at pH 8.0) or the two unresponsive ones (0.43 ± 0.14 dw/sec at pH 7.0 vs. 0.48 ± 0.20 dw/sec. at pH 8.0).

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Production and metabolism of steroids in Homarus americanus. ERNEST F. COUCH, C. A. ADEJUWON, AND S. S. KOIDE.

We have reported that the mandibular gland of the lobster contains progesterone as determined by radioimmunoassay (Couch *et al. Biol. Bull.* **155**: 433, 1978). To learn the fate of the progesterone, tritium-labeled progesterone (1×10^6 cpm) was injected into the abdominal muscle of an adult female lobster. After 1.5 hr various tissues (blood, urine, heart, green gland, hind gut, gills, mandibular gland, hepatopancreas, ovary, nerve cord, and muscle) were excised, homogenized, and extracted with petroleum ether and ethyl ether. The radioactivity in the extracts was measured. The highest incorporation of progesterone occurred in the mandibular gland (7500 cpm/mg wet wt.) and in the green gland (2750 cpm/mg wet wt.).

To determine the capability of lobster tissues to transform steroids, the mandibular gland, hepatopancreas, and ovary were incubated with ^3H -pregnenolone for 6 hr *in vitro* at room temperature. The reaction mixture was extracted with petroleum ether and ethyl ether. The extracts were pooled and dried. The residue was dissolved in chloroform:methanol (1:1) and spotted on silica gel TLC plates. The plates were developed in a mixture of petroleum ether:ethanol:water (100:80:20 by volume). The spots were visualized under UV light. By this method pregnenolone and progesterone were not separated. However, in the extract of the hepatopancreas, a unique radiolabeled metabolite ($R_f = 0.71$) was detected which was distinctly different from progesterone, testosterone, estrone, estradiol, and estriol. This UV-absorbing compound was also found in untreated hepatopancreas, but it was absent in ovary and mandibular gland. These results indicate that pregnenolone is transformed into a metabolite in the hepatopancreas under physiological conditions.

To establish a relationship between mandibular gland function and reproduction, ovaries from lobsters at varying stages of oocyte development were excised and fixed for morphological studies. Previous studies had shown the presence of small amounts of progesterone (about 0.2 ng/g of wet wt.) in immature ovaries but not in mature ovaries. In the present study, we observed that the size of the mandibular gland appears to vary during ovarian development; *i.e.*, the gland was small (about 12 mg/100 g of body wt.) during the dormant stage of egg production. It increased in size (about 25 mg/100 g of body wt.) during the time of early oocyte growth and development and receded to about 9 to 11 mg/100 g of body weight as maturation of oocytes occurred. These results suggest that mandibular gland function might be associated with ovarian development.

Supported by a grant from the George Hecht Fund.

Response specificity following behavioral training in the nudibranch mollusk Hermissenda crassicornis. TERRY CROW AND NANCY OFFENBACH.

Photopositive responses of the nudibranch mollusk *Hermissenda crassicornis* exhibit long-term behavioral plasticity that has been examined at the cellular level. The behavior is modified by an automated training procedure that stimulates the eyes and statocysts using light paired with rotation while the animals are confined to the ends of glass tubes filled with sea water. Random control procedures have demonstrated that the long-term behavioral modification is dependent on pairing light and rotation. Response latencies to move into an illuminated area are significantly increased for animals that received light paired with rotation. The increased response latencies following training are not the result of a decrease in general activity. When the same animals were tested in both light and dark after training we found that only response latencies to enter the illuminated area were increased ($P < 0.01$). Latencies to move to the end of the tubes in the dark were not significantly changed. Significant light-dark differences were not found for random control or normal untrained animals. We next examined the response latencies of animals to initiate movement towards the illuminated area at the opposite end of the tube. We found a linear relationship between the start latencies (initiation of movement toward the light) and the latencies to enter light for normal untrained animals ($r = 0.93$, $P < 0.01$). Start latencies for trained animals were correlated with the latencies to enter light before ($r = 0.97$) and after training ($r = 0.99$). We found significant increases in start latencies following training for the light paired with rotation group ($P < 0.005$) while the start latencies did not change for random control groups.

The increase in the response latencies to enter light after training can be accounted for by the significant increase in start latencies for trained animals. This specific modified behavioral response to light following training will be examined at the cellular level in intact and semi-intact preparations.

In vitro transcription in nuclei from *Arbacia punctulata*. LISA M. DAVIS.

A technique for isolating nuclei which are capable of incorporating ^3H -UTP into TCA precipitable counts has been devised. This incorporation is inhibited by the presence of α -amanitin, it is dependent upon the presence of all four ribonucleotide triphosphates, and the reaction yields a product which is Ribonuclease A sensitive. The isolation procedure yields nuclei which are not aggregated and which are free of cytoplasmic contamination. Conditions for maximizing the reaction, with regards to the divalent metal ions and the salt concentrations, were also investigated. The procedure for isolating transcriptionally active nuclei called for the presence of MgCl_2 , NP-40, and EGTA, in the homogenization buffer. The former two ingredients were important for obtaining clean, non-aggregated nuclei, and the latter for inhibiting nucleases. Nuclei from embryonic stages other than gastrula were not assayed for this transcriptional activity. However, the observation was made that nuclei isolated from embryos grown at 25°C were only 25% as active as those from embryos grown at 18°C . Following isolation, the nuclei were either assayed immediately, or stored at -20°C , with no loss of activity for at least 2 weeks.

The α -amanitin profile of this activity was determined; the reaction was inhibited 50% by both high and low levels of the toxin (100 and $5\text{ }\mu\text{g/ml}$, respectively). Whether this result is due to the absence of any polymerase III activity, or to the possibility that the enzyme is sensitive to higher levels of amanitin, was not determined at the time of this writing. However, attempts were made to optimize for activity by polymerase III, by altering the Mg^{2+} concentrations and using Mn^{2+} and KCl concentrations which typically stimulate this activity. It was determined that the time course of the reaction, as well as the amanitin profiles, were identical with either 1 mM, 5 mM, or 10 mM Mg^{2+} . (The assay mix also contained 1 mM Mn^{2+} and 150 mM KCl).

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Chemical search image: prey exposure improves selective chemical detection by a predator (Homarus americanus). CHARLES DERBY AND JELLE ATEMA.

Considered as a species, lobsters (*Homarus americanus*) are omnivores; individuals, however, can be selective in prey preferences. Experience with prey can alter prey preferences by various methods: learning to search for prey in particular areas; learning to handle prey more efficiently; and learning to detect prey. The latter case, where encounters with prey improve the predator's detection of that prey, is called search image formation. This report describes chemical search image formation in lobsters.

Behavioral responses of ten lobsters to prey body odors were observed in 2.5-m-long troughs. Body-odor stimuli of two prey species, *Mytilus edulis* (blue mussel) and *Modiolus modiolus* (horse mussel), were prepared by placing five mussels in sea water for a fixed amount of time. For each stimulus, lobsters were observed for 10 min before and 10 min after stimulus introduction. Movements of appendages associated with alerting and searching (antennules, antennae, maxillipeds, claws, walking legs) were quantified, and differences between pre- and post-introduction periods were determined by Chi-square analyses. Detection thresholds for the two stimuli were determined by randomly presenting seawater controls and log-dilutions of both odors. Effects of feeding experience on thresholds were examined by initially feeding starfish and fish to all ten lobsters. After determining thresholds, lobsters were placed in a tank with either live *Mytilus* or live *Modiolus*. After 30 days of this exposure, thresholds were again determined. Changes in responses to experienced prey odors were compared relative to changes in response to nonexperienced prey odors. Seven lobsters became relatively more sensitive to odors of experienced prey; two showed no change; one became relatively less sensitive. Wilcoxon matched-pairs signed-rank test demonstrates significant at $P < 0.05$. This

experiment therefore demonstrates that chemical detection of prey improves as a function of experience with that prey; this supports the idea that lobsters can form chemical search images.

Effects of unilateral antennule ablations on food odor orientation in Homarus americanus. DANA DEVINE AND JELLE ATEMA.

Recent electrophysiological investigations of the lobster antennule have suggested that the lobster can orient to odors by gradient discrimination through simultaneous comparison of bilateral receptor input from the two lateral flagella of the biramous antennule. Behavioral evidence supporting this hypothesis is anecdotal and scant, prompting the present study.

Lobsters were placed individually in 600-liter aquaria, where they occupied a shelter in the middle of the back wall. Two air life water flows were directed toward the center of the tank from the side walls, running continuously. For testing, a standard food odor was injected in either flow. The lobsters' behavior was observed and timed in response to this chemical stimulus. Three sets of data were obtained per lobster in the following order: untreated, after ablation of the right lateral flagellum, and after subsequent ablation of the ipsilateral medial flagellum. The following results were obtained. First, the initial direction choice during searching changed dramatically from 100% correct to roughly random upon ablation of one lateral flagellum. This illustrates the importance of lateral flagellar input for direction choice at the start of a search. Secondly, the paths followed by ablated animals when searching for the source of the stimulus were significantly more random than those of normal lobsters. Thus, lobsters missing one lateral flagellum could no longer efficiently follow the changing stimulus concentrations within the odor cloud. However, cirrus movements were never seen in ablated animals. Thirdly, ablation did not impair the lobsters' ability to detect the presence of a stimulus: the alert time did not increase. After ablation of the ipsilateral medial flagellum, there was a significant decrease in alert time which has no obvious explanation. These data give behavioral support to the hypothesis that the receptor input from both lateral flagella (perhaps from the aesthetasc hairs themselves) are used for efficient orientation to a food odor.

All the poly(A)(+)mRNA sequence complexity also occurs in poly(A)(-)mRNA in sea urchin embryos. ROGER DUNCAN AND TOM HUMPHREYS.

Polysomal RNA was purified from *Lytechinus pictus* hatched blastulae, and fractionated into poly(A)(+) and poly(A)(-)RNA by multiple passages through an oligo d(T)-cellulose column. A cDNA probe was synthesized using the poly(A)(+)mRNA as the template and AMV reverse transcriptase. The extent to which the poly(A)(+)mRNA sequence complexity also occurs in poly(A)(-)mRNA was determined by hybridizing the cDNA to both RNA populations. The complete hybridization reaction of the cDNA to its template poly(A)(+)mRNA spans about 3 decades on a log R₀t axis, indicating that a range of mRNA abundancies exists, and terminates with greater than 80% of the cDNA in hybrid form. The poly(A)(-)RNA also hybridizes greater than 80% of the cDNA, indicating that all the poly(A)(+)mRNA sequence complexity is also present in mRNA molecules containing little or no poly(A). The cDNA-poly(A)(-)RNA hybridization kinetics show that the poly(A)(-)mRNA molecules which are driving the reaction are represented in the polysomes at about the same frequency as the poly(A)(+)mRNAs. The poly(A) and oligo(A) length and content of both RNA preparations was examined by digestion with RNase followed by gel electrophoresis. Comparison of the relative amount of poly(A) in the poly(A)(+) and poly(A)(-)RNA preparations showed that greater than 90% of the mRNA molecules driving the cDNA-poly(A)(-)RNA hybridization reaction lack a poly(A) segment longer than 20 nucleotides. We conclude that a specific set of mRNA sequences which are exclusively polyadenylated does not exist in sea urchin embryo mRNA, confirming a recent *in vitro* translation comparison.

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Studies on actin mRNA-complimentary genomic DNA sequences in the sea urchin, S. purpuratus. DAVID S. DURICA, JEFFERY A. SCHLOSS, AND WILLIAM R. CRAIN.

An actin gene-containing plasmid recently isolated from *Drosophila melanogaster* (E. Fyrberg and N. Davidson, personal communication) was tested for interspecific sequence homology to sea urchin (*S. purpuratus*) DNA. The actin coding region of the cloned *Drosophila* DNA cross-reacted significantly in Southern transfer experiments using several restriction enzymes, hybridizing to a limited number of discrete bands (4-8 depending on the enzyme). Solution hybridization followed by HAP chromatography also indicated significant cross-reaction, with reassociation kinetics suggesting a homogeneous component repeated less than 10 copies per genome. Sequences which specifically cross-reacted with the *Drosophila* probe were cloned into the bacterial plasmid vector pBR322. These clones fall into two size classes which correspond to genomic Hind III fragments of 3.6 and 7.0 kb. Both clones tested positive when assayed for the ability to selectively capture actin mRNA from a total mRNA population. Both plasmids are presently being characterized by restriction and heteroduplex mapping to determine regions of homology between the two sea urchin plasmids and the *Drosophila* clone.

D.S.D. is a fellow of the Muscular Dystrophy Association. This research was also supported by NIH Training Grant #TG-HD07098.

Studies of the translational regulation of histone synthesis in Arbacia punctulata. CHRISTOPHER EARL AND TIM HUNT.

Histone synthesis in the somatic and embryonic cells of a variety of organisms is coupled to DNA replication. Control is exerted at the levels of transcription and translation, but the molecular basis of the regulatory mechanisms is unknown. We decided to test the hypothesis that histones bind specifically to histone mRNAs and block their translation. We prepared histones and histone mRNA from hatching blastulae of *A. punctulata* and added both to reticulocyte lysates. We found that histones did indeed inhibit protein synthesis, but that this inhibition was not specific for histone mRNA.

Incubation of *A. punctulata* embryos in 1 mM hydroxyurea inhibits both DNA synthesis and histone synthesis within 30 min, as measured by incorporation of radiolabeled thymidine and methionine or lysine, respectively. We therefore examined the stability of translatable histone mRNA *in vitro*. We prepared cytoplasmic extracts from four hour blastulae, untreated or treated for 90 min with 1 mM hydroxyurea. After homogenization and centrifugation, the postmitochondrial supernatants were equilibrated with reticulocyte salts on a Sephadex G-50 column. We mixed 3 volumes extract with 1 volume reticulocyte lysate to boost *in vitro* translation. Histone synthesis was significantly reduced in the hydroxyurea extracts after treatments longer than 1 hr, but it is not clear whether this result is due to active degradation of the histone messages or to a halt in their transcription and an unaltered rate of nonspecific turnover.

Supported by NIH training grant TG-HD07098.

Temperature-dependent timing of mitosis and cleavage in Lytechinus variegatus. ANDREW EISEN AND SHINYA INOUÉ.

At a constant temperature individual embryos of the sea urchin *Lytechinus variegatus* divide at intervals with precision far greater than apparent in a batch of sibling embryos. A series of events recur with the exact same interval for the first four divisions. Taking the completion of cleavage as unity, the events occur at the following fractions of the cleavage interval: nuclear envelope breakdown (0.45), spindle formation (0.50), metaphase (0.06), anaphase-A onset (0.71), anaphase-B onset (0.79), cleavage onset (0.85), cleavage 50% completed (0.93). The fractions remain constant over the full range of temperatures which permit development (17-33° C). Thus a plot of division events vs. time yields a straight line for a single embryo at a given temperature. All lines intercept the abscissa just as the "nuclear streak" appears. Between 17° C and 27° C the slopes increase with temperature with a Q₁₀

of ca. 2. While the division schedule suggests a continuously operating temperature-dependent "clock", such is not the case. When the temperature is raised from 17 to 27° C, the schedule immediately shifts to the new rate. However, when the temperature is dropped during the second division from 27 to 17° C, the rate does not immediately shift. Rather, the shift is variously delayed. The delay can be as great as 23 min when the embryo is chilled very early after cleavage. Before metaphase the delay progressively decreases as though cells enter the new schedule at around metaphase regardless of the stage at chilling. Thus portions of the cell-division cycle (ca. 0.0-0.65) appear insensitive to temperature drops (from 27-17° C) and their rates are pre-determined by the temperature at an earlier time.

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Population density, size distribution and home range of the American Eel (Anguilla rostrata) in the Great Sippewissett salt marsh. TIM FORD AND EVAN MERCER.

The American Eel is increasing in commercial importance, yet little demographic or behavioral information is available for eels in coastal and inland habitats. Local eel populations were studied by trap-recapture methods along 600 m of tidal creek in Great Sippewissett marsh, Falmouth, Massachusetts, during summer 1979. The creek system varied in bottom topography and sediment type and encompassed both high and low marsh. Conical minnow traps (Gee-Gee Co.) were used which captured eels larger than 15 cm (standard length); the largest eel trapped was 62.5 cm. Eels were fin-clipped, using a site specific code. Additionally, animals were measured, and body blemishes noted. Intersection of body length, blemishes or marks, and fin notches allowed individual eels to be distinguished. A total of 300 eels were marked and 67 individuals recaptured; some of these several times. Capture-recapture data was analysed with Jolly's model, which estimated a population of 350 eels in the 600-m creek system. Of this population small eels (less than 30 cm) dominated captures in the narrow, soft-bottomed creeks of the high marsh, and larger eels (greater than 30 cm) in the broader, soft mud to sandy bottomed creeks of the low marsh. Distances travelled by recaptured eels gave an indication of home range. Two eels dispersed over 200 m; the majority dispersed over much less distance (average 38 ± 54 m), which suggests a small home range. Little movement was observed for eels greater than 40 cm and the largest eels were commonly recaptured several times at specific sites. The results indicate large eels may establish territories in low marsh, restricting small eels to high marsh creeks.

Studies by fluorescence of protochlorophyll(ide) and its phototransformation in dark-grown Euglena gracilis var. bacillaris. MARCIA A. FREY, RANDALL S. ALBERTE, AND JEROME A. SCHIFF.

Intact cells show a fluorescence emission peak at about 635 to 638 nm from excitation of protochlorophyll(ide) (Pchl(ide)) 634; no emission peak is seen in the 650 to 660 nm region. Emission peaks at 712 and 730 nm, also due to Pchl(ide) excitation, may represent aggregates. Emission at 642 nm appears to be due to protophenophytin/phorbide (Pphea) based on excitation spectra. On exposure to light, the 638 nm peak decreases and chlorophyll (Chl) and, perhaps, pheophytin a (Phea a) emission appears, but the 712 and 730 nm emissions do not change. Acetone extracts transferred to diethyl ether show emission bands at: 630 nm (Pchl(ide): excitation maxima at 435, 535, and 571 nm); 590 nm (Mg protoporphyrin IX (monomethyl ester?): excitation maxima at 417 and 551 nm); 512 nm (phytofluene: excitation maxima at 333, 348, and 368 nm); and in the 642 nm region (Pphea: excitation maxima at 417, 525, 565, 585, and 638 nm). Acidification and washing of the ether yields more Pphea and less Pchl(ide) and phytofluene. Extracts of light exposed cells show similar spectra but Pchl(ide) is diminished and an emission at 673 nm appears due to a mixture of Chl and Phea a based on excitation spectra. On acidification, this emission decreases and appears to be due solely to Phea a. As found previously by absorption, Pchl(ide) 634 is present and phototransformable to Chl but Pchl(ide) 650 is undetectable. Pphea is detected in intact dark-grown cells and is probably not an extraction artifact; Pphea to Pchl(ide) ratios of about 10:1 were found previously by absorption spectroscopy of extracts. Since Phea a is

found after light exposure, one possibility of many is a photoconversion of Ppheo to Pheo a. The detection of Mg protoporphyrin IX suggests a control point just after this compound in the biosynthetic pathway of Chl. Fluorescence has permitted the detection of phytofluene, a precursor of carotenoids, in these cells.

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Electron spin resonance studies of protein-methylglyoxal complexes and model systems. PETER R. C. GASCOYNE.

The interaction of methylglyoxal with bovine serum albumin, collagen, lysozyme and casein has been studied using electron spin resonance spectroscopy. A radical signal centered at $g = 2.005$ accompanied the reaction of methylglyoxal with the proteins studied and this radical was found to be stable to drying. If the lysine residues in the protein were first blocked by dimethylation, however, no such radicals could be induced by methylglyoxal treatment, indicating that the lysine sidechains of the protein were involved in the radical formation. This observation of the primary importance of lysine is consistent with the spectroscopic findings of Pethig and McLaughlin (1979, *Biol. Bull.*, 157: 388).

In order to further understand the interaction between methylglyoxal and the protein lysine sidechains, a simple model, methylamine, has been investigated using a computer controlled stop-flow technique. In this way the development of the various radical species accompanying the mixing of molar methylglyoxal and methylamine solutions has been followed from 0.01-100 sec. Under a nitrogen atmosphere a singlet centered at $g = 2.005$ appeared in about 2 seconds followed by the development in a few tens of seconds of a septet with 8.5 Gauss splitting, and later by other radicals. The addition of sodium ascorbate greatly enhanced and accelerated the formation of the observed radicals and the presence of at least one additional radical species was detected after 20 sec. This observation of the importance of ascorbate is in agreement with the suggestion of Szent-Györgyi (1979, *Biol. Bull.*, 157: 398) that the ascorbate molecule tends to facilitate the process of molecular charge transfer in methylglyoxal-amine systems.

Is there tubulin in spirochaetes? SHARON GREENBERG.

The theory of the exogenous origin of microtubules in eucaryotic cells is based on the hypothesis that an early symbiotic relationship between primitive procaryotes, specifically spirochaetes, and eucaryotic cells, led to the evolution of motile organelles of existing eucaryotic organisms. This theory predicts the existence of tubulin-like proteins in spirochaetes.

The presence of tubulin in spirochaetes was investigated using the free-living, facultatively aerobic, halophilic spirochaete, *Spirochaeta halophila*. Microtubule-like cytoplasmic fibrils have previously been demonstrated in this bacterium by electron microscopy. Cultures of *S. halophila* were analysed for tubulin-like proteins by comparison of migration patterns on two polyacrylamide gel systems of whole-cell spirochaete protein fractions with an axoneme-derived tubulin standard prepared from sea urchin sperm. On 7.5% Laemmli slab and tube gels, there were protein bands in the spirochaete sample which apparently comigrated with α - and β -tubulin in the axoneme standard. On Fairbanks gels, the axoneme-derived tubulin standard produced the predicted single band at 55,000 MW. The putative spirochaete tubulin, however, ran as multiple bands; in some cases lower than the axoneme tubulin standard.

It would seem that on the basis of the Laemmli gel system there are proteins in the spirochaete sample with mobilities similar to that of authentic tubulins. When the same sample is run on Fairbanks gels, however, the apparent homology between the proteins breaks down. A conclusive statement cannot be made at this time, based on the results obtained from polyacrylamide gel electrophoresis, concerning the presence of tubulin-like proteins in the spirochaete *S. halophila*.

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Structural changes associated with increased coupling resistance in the septate axon of the crayfish. ROBERT B. HANNA, GEORGE D. PAPPAS, AND MICHAEL V. L. BENNETT.

Increased coupling resistance was brought about by mechanical injury to identified segments of the lateral septate axon in the ventral nerve cord of the crayfish. The axons were fixed at specific time intervals following injury and prepared for electron microscopy. Both freeze-fracture replicas and thin sections were utilized. The intramembranous particles of the control electrotonic junctions are 12.5 nm in width, have a center-to-center spacing of 20 nm, and are organized in a loose hexagonal array. Following injury, the intramembranous particles of the identified high resistance electrotonic junctions were found to lose their organization and become more widely spaced. In some experiments the particle spacing and organization remained unchanged for as long as 45 min following injury. Thin sections corroborated the findings from freeze-fracture replicas.

Following injury there is an increased density of the axoplasm and an increased number of cellular organelles in the uninjured axon proximal to the junction. Concomitant with the increased cytoplasmic density, there is a mobilization of the Schwann cells which become interposed between the injured and uninjured segments (Pappas, Asada, and Bennett, 1971, *J. Cell Biol.*, 49: 173-188). The mobilized Schwann cells develop increased extent of anastomosing tubular networks, which is continuous with their plasma membrane. Eventually the junctional complex is apparently internalized in the axoplasm of the uninjured axon.

*Absorption properties of *Platymonas* sp. Rey 2 containing a high proportion of chlorophyll b.* B. A. HAYHOME, JEROME A. SCHIFF, ROBERT R. L. GUILLARD, AND RANDALL S. ALBERTE.

Platymonas sp. Rey 2, a marine green prasinophyte alga, recently obtained in axenic culture (from Venezuela), has 55% of its total chlorophyll (a + b) in the form of chlorophyll b as determined by absorption spectra *in vivo* and *in vitro*, by chromatographic separation of the pigments followed by spectrophotometric determination and by fluorescence excitation spectra of the whole cells. Absorption spectra of the intact cells show a greatly increased absorption in the chlorophyll b region (653 nm) and a sharper drop on the long wavelength side of the chlorophyll a peak compared with organisms having the usual levels of chlorophyll b (e.g., *Chlorella pyrenoidosa* Emerson strain) or low levels (*Euglena gracilis* var. *bacillaris*). The total absorption cross section in the red region of the spectrum, however, is very similar for all three types of organism. This suggests that some antenna chlorophyll a of system 2 of photosynthesis is replaced by increased chlorophyll b which maintains the effective cross section for absorption but moves the absorption region to somewhat shorter wavelengths in the red in these organisms; this may be advantageous in competition for light with organisms having normal chlorophyll b levels. Plating efficiencies of 100% have been achieved for this organism and the conditions for treatment with chemical mutagens have been worked out; chlorophyll-b-deficient mutants would be of help in learning more about the biosynthesis of chlorophyll b and its role in photosynthesis.

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*The major protein in *Spisula* sperm nuclei is a protamine.* LOUIS HERLANDS AND JUAN AUSIO.

When SDS gels are used to analyze the 0.25 N HCl extractable proteins isolated from *Spisula solidissima* sperm nuclei, a histone pattern similar to that of somatic cells is found. But in comparison with *Spisula* embryos there are few other proteins present, presumably because of the lack of contaminating cytoplasm and ribosomes in the sperm system. This

observation has led others to believe that *Spisula* is a typical histone-type sperm that would be a good model system for studying both nucleosome structure and the fate of sperm nuclear proteins after fertilization. However, when the same proteins are run on HOAc/urea gels, we see a new band which corresponds to an unusual protamine-like protein which is insoluble in SDS. More than 80% of its amino acid residues are Lys, Arg, Ser, or Ala. This new band is the major component; only small amounts of histones are present. These histones most likely come from immature sperm since first, there are several acetylated forms of H4; and secondly, gel scans indicate that the amount of histone in relation to protamine is variable. Digestion of *Spisula* sperm nuclei with micrococcal nuclease indicates that *Spisula* sperm chromatin is primarily *not* in the nucleosome configuration. Only small amounts of 140 BP protected DNA fragments are generated at early digestion times. The amount of this particle does *not* increase as digestion proceeds. The origin of this particle may either be immature sperm or perhaps a subset of chromatin in the mature sperm that is still organized with histones. Even though *Spisula* sperm contains predominantly protamine, an extremely stable PCA soluble limit, similar to somatic chromatins, is reached. But unlike chromatin, the DNA fragments are heterogeneous in size.

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Light-harvesting pigment-protein complexes from brown algae and diatoms: implications for the organization of the photosynthetic unit. D. L. GUSTAFSON, A. L. FRIEDMAN, M. S. RUDNICK, H. LYMAN, AND R. S. ALBERTE.

The pigment-protein complexes of three macrophytic brown algae, *Laminaria saccharina*, *Chorda filum* and *Pylaiella littoralis* and the diatom *Skeletonema costatum* were examined with respect to the organization of the photosynthetic unit (PSU) by fluorescence emission and excitation analysis and polyacrylamide gel electrophoresis of membrane detergent extracts. The fluorescence characterization of whole thalli, chloroplast membrane fragments, detergent solubilized thylakoids and gel electrophoretic zones were used to determine the paths of energy transfer among the light-harvesting pigment of the PSU. Pigment composition, pigment-protein biochemistry and physiological data support two paths of energy transfer. One path involves the coupled transfer of excitation energy from chlorophyll (Chl) c to Chl a, while the other involves energy transfer from the carotenoid fucoxanthin to Chl a and not to Chl c. Polyacrylamide gel electrophoresis of SDS solubilized thylakoid membranes of these algae results in two major pigment-protein zones. The slowest migrating zone shows fluorescence and absorption characteristics of a pigment-protein containing Chl a and Chl c with energy transfer from Chl c to Chl a. The other major zone shows spectral characteristics of a pigment-protein complex containing Chl a and fucoxanthin with energy transfer from fucoxanthin to Chl a. Physiological data obtained during nitrogen starvation of the diatom *Skeletonema* demonstrates a specific loss in fucoxanthin and Chl a. Accompanying these pigment losses are decreases in PSU size and a reduction in photosynthetic rates. In contrast, supraoptimal nitrogen levels lead to a cellular increase in these pigments and the pigment-protein complexes. The increase in pigment-protein may represent a nitrogen storage mechanism for these cells, and as such implies an opportunistic strategy for their adaptation to fluctuating nutrient levels.

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Water relations and photosynthetic characteristics of the tall and short ecophenes of Spartina alterniflora. ARTHUR M. B. HOGAN, DAVID MAUZERALL, AND RANDALL S. ALBERTE.

The tall ecophene of *Spartina alterniflora* in Sippiswissett Marsh is inundated for the greatest part of the tidal cycle while the short ecophene is infrequently inundated. The present

investigation sought to account for the morphological differences by examining the water relations, morphological features, protein content and photosynthetic characteristics of the two ecophenes. All parameters were replicated at least ten times and data was significant to at least the 95% level. It was found that the short form was more succulent (41% greater) and heavier (18% greater) than the tall form. Both forms had similar leaf widths. The relative water content of the short form was lowest at midday during a high tide cycle and hence the short form was more water stressed. At pre-dawn the short form recovered to hydration levels similar to the tall form. Protein content per unit leaf area was 34% higher and 20% higher on a dry weight basis in the short form, strongly suggesting that nitrogen is not limiting growth of this ecophene. Leaf chlorophyll (Chl) content is similar ($2.9-3.2 \times 10^{-4}$ mg/mm²) in the two forms when expressed per unit leaf area but 15% greater in the tall form when expressed on a dry weight basis. The Chl a:b ratios for both forms were 3.1 ± 0.1 . Photosynthetic rates of the short form measured by oxygen evolution were 26% greater when expressed on either a Chl or an area basis than the tall form. Furthermore the photosynthetic unit (PSU) size in the short form was about 40% smaller than in the tall form. Based on the differences in leaf thickness, the short form possessed about 30% more PSUs per unit area as a result of the greater amount of tissue per unit surface area. The greater numbers of PSUs per unit area fully explains the higher photosynthetic rates per area in this form. The alterations in the packaging of the photosynthetic machinery probably represents an adaptive response to a more stressful environment of higher salinity and lower water availability. In addition, photosynthetically competent protoplasts were isolated in good yield from both ecophenes which will allow for further detailed examinations of these ecophenes.

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Brush border calmodulin: A structural protein of the microvillus core. CHRISTINE L. HOWE AND MARK S. MOOSEKER.

We are investigating the molecular basis for calcium regulation of microvillar motility in the brush border of intestinal epithelial cells. Results indicate that calmodulin is present in brush borders isolated from chicken, and that it is a major constituent of the microvillar filament bundle. Calmodulin is prepared by boiling suspensions of either Triton-treated brush borders or demembrated microvilli. No calmodulin is detected in the solubilized brush-border membrane fraction. The supernate derived from boiled microvilli contains one major polypeptide identified as calmodulin by first, comigration with brain calmodulin on SDS gels and on alkaline urea gels, where both proteins undergo a shift in mobility with Ca²⁺ present; and secondly, 4-fold activation of phosphodiesterase in the presence but not absence of calcium. The molar ratio of calmodulin to actin is approximately 1:10 in the intact brush border and increases to 1:3 in the isolated microvilli, indicating that calmodulin is predominantly localized in this structure. Calmodulin remains tightly associated with the microvillus core in the presence or absence of calcium. Furthermore, calmodulin does not bind to muscle F-actin *in vitro*, indicating that the binding of calmodulin to the microvillus core may involve protein(s) other than actin. These results suggest an intimate association between the microvillar actin filament bundle and the regulatory functions of this multifaceted protein in the brush border. Examples may include regulation of actomyosin interactions responsible for microvillar movement and calcium transport.

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Fairy rings: membrane particle arrays present during early stages of de novo ciliogenesis in Tetrahymena. L. A. HUFNAGEL.

During a study of membrane-cytoskeletal interactions related to morphogenesis in *Tetrahymena*, freeze-fracture EM was used to characterize membrane structural changes preceding formation of somatic cilia in log phase cells and oral cilia in cells synchronized for oral replacement. During early neogenesis of somatic cilia, a ring of irregularly spaced 100 to 130 Å particles was observed in the E-face of the plasma membrane. The diameter of the ring was

similar to that of cross-fractured ciliary axonemes, and a central group of 2-9 100 to 130 Å particles usually was seen within the ring. Ring particles numbered from 20 to 90, with an inverse relationship to the number of central particles. The rings resembled mushroom rings found in lawns after rainstorms; hence the name "fairy rings." Unlike the E-face, the P-face of the plasma membrane was evenly studded with small particles, with no apparent arrangement. Fairy rings were associated with specializations in membrane topography. Based on the observations, a tentative sequence for ring formation, origin of the ciliary neck-lace (a membrane specialization of the mature cilium) and concurrent topographical changes has been developed. According to this sequence, the central particles and parasomal sac (a basal body-associated plasma membrane invagination) appear earliest and may be intimately involved in positioning of basal bodies, while the fairy ring develops later, perhaps under the influence of the basal body. The fairy ring then migrates into the basal region of the nascent cilium, to become the ciliary necklace. These ideas are currently under further investigation by means of TEM. In contrast with these observations, fairy rings have not been observed during neogenesis of oral cilia. This supports the idea that the rings do not assist in the positioning of basal bodies and also suggests that they are not necessary for growth of cilia.

Supported by a Marine Biological Laboratory Steps Toward Independence Fellowship.

Structure of aggregatoin factor on sponge cells and in gels. SUSIE HUMPHREYS AND T. S. REESE.

Aggregation factor has been visualized on aggregating *Microciona prolifera* cells and in factor gels by electronmicroscopy of specimens rapidly frozen and then either freeze-substituted and thin-sectioned, for freeze-fractured, deep-etched, and replicated without chemical fixation or cryoprotectant. Each factor macromolecule, a glycoprotein (MW 18×10^6 daltons), is a ring from which radiate several side chains which give it a "sunburst" shape which is well preserved by our freezing methods. Thus, the relationships of the factor molecules with each other and the cell surfaces of aggregating cells can be directly visualized. Preliminary examination of factor alone gelled in 10 mM CaCl_2 showed many images suggestive that factor retains its sunburst configuration in gels and that contact between side arms is the rule. Chemical dissociation of sponge tissue by divalent cation depletion extracts aggregation factor but cells aggregate rapidly when divalent cations and aggregation factor are restored (T. Humphreys). Replicas of cells aggregated in factor showed factor molecules over much of their surfaces, suggesting that factor "receptors" may be evenly distributed over the whole cell surface. In sections of reaggregating cells we could sometimes see the circular backbone sandwiched between two cells; some side chains contacted one cell and some from the same molecule contacted an adjacent cell. Our results suggest that cell-recognition sites are localized in the factor side chains and that the entire connection between cells could be made by single macromolecule bridges. Most significantly, our results demonstrate that factor could function by actually forming bridges between cells.

This work was supported by NSF grant PCM 78-09309 and by IRP, NINCDS. Purified factor was provided by Tom Humphreys.

Tubulin antibody induces microtubule depolymerization in vivo and in vitro. SHINYA INOUE, KEIGI FUJIWARA, AND ELAINE D. PAPAFRANGOS.

Antibodies can be used for cytochemically localizing antigens or for functionally blocking reactions involving the antigen. We now find that a tubulin antibody (Anti-TL) can also depolymerize labile microtubules. The antibody was prepared against tubulin paracrystals induced in sea urchin *Strongylocentrotus purpuratus* eggs by vinblastine. Upon micro-injection of ca. 0.4 nanoliters of Anti-TL solution (containing ca. 150 mg/ml of immunoglobulin), the meiotic metaphase arrested spindle in the oocyte of *Chactopterus pergamentaceous*, and the mitotic cleavage spindles and asters in *Lytechinus variegatus*, substantially lose their birefringence and grow smaller in minutes. The reaction resembles cold or colchicine treatment. Spindle birefringence and size are not appreciably reduced by injection of 100 mM KCl, buffer solution alone (100 mM KCl, 10 mM phosphate buffer pH 6.9, 0.02% Na-azide),

or pre-immune IgG in buffer; if the birefringence decreases it recovers, although some cells injected with large volumes of IgG (with or without Anti-TL) blebbed extensively and lysed after approximately half an hour. Mouse and embryonic chick brain microtubules assembling *in vitro* are also depolymerized by Anti-TL, but again not by pre-immune IgG or buffer alone. The rise of specific viscosity ceases and decays exponentially as soon as Anti-TL is added to the polymerizing solution (2.5–3 mg/ml tubulin, 0.1 M PIPES, 1 mM DTT, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 4 M glycerol). We do not yet know whether the Anti-TL depolymerizes microtubules by shifting the microtubule-subunit equilibrium, or by directly attacking the microtubules themselves.

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Field and laboratory observations of lobster mating behavior. ELISA KARNOFSKY
AND JELLE ATEMA.

Courtships and mating behavior of the lobster (*Homarus americanus*) have been observed in our laboratory in large naturalistic aquaria. Field observations are now being made to compare laboratory observations to behavior in the natural environment. In the laboratory, dominant males establish stable residences and regularly displace other individuals from their burrows. There is female mate selection: females approach the dominant male burrow 1 to 5 days premolt, exhibiting ritualized behavior as do males during these entrance ceremonies. The female may leave the burrow periodically, chasing other lobsters from the area. This may be part of a generally observed premolt aggression peak. The male often lunges at the opening, preventing the female from leaving. Approximately 20 min after the female molts, the male mates with her. He then feeds on and later rejects the molt shell. Cohabitation without leaving continues for 1 to 5 days. Females may leave for short excursions before leaving permanently. Mating in the field was observed by direct and indirect clues. Two lobsters were discovered in a large burrow. The male had previously been tagged and the female had no crusher claw. This couple was observed for 8 days during which the female molted as indicated by a regenerated crusher; also, a molted seizer claw was found outside the shelter. Male entrance ceremonies were performed, as was blocking of the entrance. On the 8th day the female disappeared. The same day a tagged female appeared and cohabited for 7 days. She molted between the 1st and 3rd day. During this time the male was seen evicting another male from a nearby burrow.

This study shows that observations in naturalistic settings contribute continuity to scant and difficult-to-obtain field observations, while field studies modify and add reality to still rather artificial laboratory observations in large aquaria.

Properties of polymerizable tubulin from isolated Spisula spindles. THOMAS C.
S. KELLER III AND LIONEL I. REBHUN.

Spindles were isolated from activated eggs of the surf clam *Spisula solidissima* in a buffer containing 3.4 M glycerol, 100 mM PIPES, 1 mM $MgCl_2$, 5 mM EGTA, 1% NP-40 pH 6.8. Tubulin was recovered from the isolated spindles by washing them free of the glycerol into a buffer containing 100 mM PIPES, 1 mM $MgCl_2$, 5 mM EGTA pH 6.8 and placing them on ice until birefringence disappeared. The nonbirefringent spindle remnants were pelleted leaving the cold depolymerized spindle tubulin in the supernatant. Upon addition of GTP (to 1 mM) and warming to 24 or 37° C, the spindle tubulin polymerized into microtubules detectable by an increase in light scattering and by the appearance of birefringent strands. The reassembled spindle tubulin was further purified through two additional cycles of temperature dependent assembly-disassembly at either 24 or 37° C. After three cycles, the spindle tubulin cycled at 37° C contained predominantly tubulin, small amounts of a high molecular weight component (which differed in mobility from *Spisula* axoneme dyneins and bovine brain HMW's) and a doublet approximately 33,000 daltons. Tubulin cycled at 24° C contained at least 3 additional proteins normally lost at 37° C. Polymerization of the cycle-purified spindle tubulin was both concentration- and temperature-dependent with critical

concentrations of 0.09 mg/ml at 37° C and 0.24 mg/ml at 24° C. To determine whether the associated proteins were necessary for polymerization, they were removed by DEAE-cellulose and phosphocellulose chromatography. The resulting purified tubulin polymerized with critical concentrations of 0.11 mg/ml at 37° C and 0.28 mg/ml at 24° C, not significantly different from those obtained with cycle-purified tubulin containing associated proteins. Thus *Spisula* spindle tubulin polymerizes at physiological temperatures (24° C) and the extent of this polymerization is independent of associated proteins.

Preparation and characterization of phagosome membranes from Arbacia punctulata embryo cells. DAVID KEW, CHRISTINE KAZNOWSKI, AND JAY BROWN.

Phagosomes from dissociated *Arbacia* embryos at eight-cell and hatched blastula stage were characterized by scanning and transmission electron microscopy, by enzyme assays, and polyacrylamide gel electrophoresis. Phagosomes were isolated after allowing the single cells to phagocytize latex beads.

Cysteine/papain treated eggs were grown to eight-cell stage for dissociation in Ca^{2+} , Mg^{2+} -free sea water. Untreated eggs were grown to hatched blastula for dissociation— Ca^{2+} -free sea water washes, then shearing in sucrose/citrate/EDTA. Washed cells were incubated with 0.7 micron latex beads—3 to 4 hr for eight-cell stage, and 6 to 8 hr for blastula. Washed cells were lysed in hypotonic buffer and lightly homogenized. Phagosomes containing beads were isolated on a sucrose step gradient spun at $100,000 \times g$ for 90 min, at the 15 to 20% interface. Washed phagosomes contained an average of 0.6% of the homogenate protein. SEM series show progressive development of bead-size lumps in the blastula cells, and TEM sections show beads deep in the cells. Lactoperoxidase-catalyzed ^{125}I -labeling showed some major different proteins labeled on living cells compared to washed phagosomes, when the proteins were separated by PAGE on Matsudara gels. Membrane enzymes assayed were: cytochrome c oxidase for mitochondria, NADH diaphorase for smooth ER, acid phosphatase for lysosomes, 5'nucleotidase and phosphodiesterase for plasma membrane. The non-plasma membrane markers were greatly reduced in the phagosomes relative to the homogenate, except acid phosphatase was slightly increased in the blastula cells. This increase is probably due to phagosome-lysosome fusion after the long incubation. 5'nucleotidase was assayed for eight-cell stage, at 7-fold phagosome enrichment. Phosphodiesterase was assayed at blastula stage, at 10-fold phagosome enrichment. The method shows a good yield and purification of membrane recently phagocytosed. It offers an opportunity to investigate properties and changes in a developing system, and as a non-developmental system to investigate transport, turnover, or pharmacological effects.

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Adaptation of bacteria to rapidly changing environmental conditions. DAVID KIRCHMAN AND RALPH MITCHELL.

We used tidal flow in the Great Sippewissett salt marsh, Massachusetts, as a model system to determine the response of bacteria to rapidly changing environmental conditions. The number of bacteria was measured over two tidal cycles using the acridine orange direct count method (AODC). The total number of free bacteria was approximately $2.0 \times 10^6/\text{ml}$ at low tide. As the tide came in, total numbers decreased to a minimum at high tide of $1.0 \times 10^6/\text{ml}$. The number of free bacteria increased again to approximately $2.0 \times 10^6/\text{ml}$ as the tide left the marsh. We measured growth rates of bacteria from Buzzards Bay and from the marsh at low tide, in filtered sterilized ($0.22 \mu\text{m}$ Millipore) water from Buzzards Bay and from the marsh. Growth rates were determined by measuring changes in bacterial numbers with AODC over 10 hr in 300-ml BOD bottles incubated in the marsh. Marsh bacteria grew in Buzzards Bay water at lower growth rates than in marsh water. Minimal growth of Buzzards Bay bacteria in Buzzards Bay water was detected in the 10 hr experiment. Bacteria from Buzzards Bay did grow in marsh water, but this growth does not appear to be fast enough to account for the observed changes in bacterial numbers over a tidal cycle at Great Sippewissett. While bacterial numbers doubled in 6 hr from high to low tide,

significant growth by bacteria from Buzzards Bay in marsh water was not evident until the eighth hour. The fate of marsh bacteria carried into Buzzards Bay is unclear over a longer time scale. Bacteria from Buzzards Bay do not appear to adapt to the changing environmental conditions in the marsh rapidly enough to account for the pattern in bacterial numbers over a tidal cycle. A fresh inoculum of bacteria from the sediment at each tidal cycle may explain the large population at low tide.

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How photoisomerizable azobenzene compounds affect acetylcholine receptors of skate muscle. M. KROUSE, H. LESTER, AND M. WEINSTOCK.

Previously, we have studied the effects of azobenzene compounds on *Electrophorus* electroplaques. This work extends those investigations to the skate, *Raja erinacea*. Photoisomerizable drugs were applied to the depressor rostri muscle. Effects of these drugs on endplate potentials and endplate currents (recorded under voltage clamp) were examined. *Trans*-Bis-Q is a potent agonist in the eel electroplaque ($K_D = 150$ nM at -150 mV). A $30 \mu\text{M}$ solution of Bis-Q containing predominantly the inactive *cis* isomer was added to the voltage clamped muscle. Then, a light flash increased the concentration of the *trans* isomer from about 5 to $11 \mu\text{M}$ and thus induced a net inward current of 15 nA. This increased inward current was, however, transient even though both isomers are stable in the dark. Ten μM 2BQ, a competitive antagonist (in its *cis* isomer) on the eel electroplaques ($K_I = 150$ nM) reduced the amplitude of the endplate current to about 25% of its control value. This effect was seen with both the *cis*- and *trans*-configurations of 2BQ. Ten μM benzyl Bis-Q inhibits the response to carbachol in the eel but in the skate muscle the effect is to increase the amplitude of the endplate potential and to prolong its decay, suggesting that it is a cholinesterase inhibitor. A small antagonist effect would not have been seen. EW-1, a local anesthetic (in its *cis* isomer) on the eel electroplaque ($K_I = 25 \mu\text{M}$) has no effect on endplate currents with concentrations as high as $100 \mu\text{M}$. While there are differences between eel electroplaque and skate muscle in regard to drug action and potency, the observations reported here serve to demonstrate that photoisomerizable compounds may be profitably used on preparations other than *Electrophorus*.

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Characteristics of the photosynthetic unit in macrophytic red algae. T. A. KURSAR, D. MAUZERALL, AND R. S. ALBERTE.

Phycobilisomes were isolated from field-collected *Griffithsia* sp. and *Champia parvula*. Excitation of intact phycobilisomes with 500 nm light results in a small fluorescence emission at 576 nm from phycoerythrin and a large peak at 672 nm. *Griffithsia* sp. phycobilisomes in 0.65 M Sorensen's buffer have an uncorrected sedimentation coefficient of 83S.

The *in vivo* fluorescence of wild type *Gracilaria tikvahiae* and an orange mutant (P-7-1, obtained from J. van der Meer, NRC, Halifax, Nova Scotia) was also measured. Excitation of chlorophyll *a* with 430 nm light resulted in emission at 696 nm in both wild type and the mutant. Excitation of phycoerythrin at 550 nm in the wild type resulted in phycoerythrin emission at 577 nm, an unidentified emission at 644 nm, and a prominent shoulder at 683 nm, whereas the mutant (P-7-1) showed a decreased 644 nm emission, almost no 683 nm emission and an emission band at 700 nm. The excitation spectrum of the 690 nm emission includes phycoerythrin and a large contribution from phycocyanin and allophycocyanin in the wild type, whereas for P-7-1 the 690 nm fluorescence is principally excited by phycoerythrin with a small contribution from phycocyanin and no apparent contribution from allophycocyanin. The chlorophyll content of the wild type and P-7-1 are the same, but P-7-1 is low in carotenoids. The growth rate of P-7-1 is about half that of the wild type. Our preliminary conclusion is that the light harvesting system of photosystem I is normal in P-7-1 and that the lesion

has altered the phycobilisome or the light harvesting chlorophylls and carotenoids associated with photosystem II (or both). These results also suggest that in P-7-1 excitation energy transfer may go directly from phycocyanin to chlorophyll *a* and bypass allophycocyanin.

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Slow changes in the magnitude of the potassium current associated with changes in the internal perfusion solution in squid axons. DAVID LANDOWNE AND VIRGINIA SCRUGGS.

Experimentally we find it difficult to alter the internal potassium concentration in internally perfused squid giant axons. Apparently there is no inert replacement monovalent cation which does not itself reduce the potassium current. When we reduced the internal potassium, replacing it with sucrose, we find the magnitude of the potassium current declines in two phases. There is a rapid decline during the first 1 to 2 min which corresponds with the time required to change the internal solution. This is followed by a slow decline over tens of minutes. This is reversible, on switching back to a high potassium solution the current increases in two phases. The relative proportion of the two phases varies. We think this is associated with damage to the interior of the axon, with less damage favoring more slow phase. Prolonged treatment with pronase removes the slow phase.

From these current measurements it seems that the residual axoplasmic lattice retains some of the potassium and only slowly equilibrates with the perfusion fluid when no cation is provided to exchange for potassium. It will similarly retain sodium or TMA. When the internal solution was changed from one containing sodium and potassium to one containing potassium only, and with sugar replacing sodium, the current-voltage curve retained the negative conductance region associated with the presence of sodium. A 1-min rinse with high potassium followed by return to low potassium removed the negative conductance.

Phosphocellulose purification of dogfish and skate brain tubulin. GEORGE M. LANGFORD, LASCELLES E. LYN-COOK, AND DANIEL ROBBINS.

Dogfish shark and skate brain tubulins were purified first by two cycles of a temperature dependent assembly-disassembly procedure and then by phosphocellulose (PC) column chromatography. The cycled tubulins were capable of spontaneous assembly but the phosphocellulose purified tubulins were not. The cycled tubulins, nevertheless, contained no high molecular weight, microtubule associated proteins (MAPs). The high molecular weight proteins present in the supernatant and pellet fractions obtained after each of the assembly-disassembly steps were determined by sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE). The gels of the initial extract show two major high molecular weight protein bands of approximately 280,000 and 320,000 daltons. At least two minor bands in the molecular weight range of 300,000 daltons could also be seen. One of the two major proteins, the 280,000 daltons protein, is lost in the first cold pellet. The 320,000 dalton protein which co-migrates with ciliary dynein 1, co-purifies with tubulin through the largest number of steps. Sometimes it is retained after completion of two cycles of purification but most often it too is lost in an earlier step. The twice cycled tubulins free of high molecular weight proteins were put on a phosphocellulose column. Because phosphocellulose sequesters Mg^{2+} , this ion was immediately added to each of the column fractions. The fractions containing tubulin were combined and concentrated by a Millipore immersible ultrafilter. Phosphocellulose tubulin, in the range of 0.5 to 1.0 mg/ml, did not spontaneously assemble. When mixed with cycled tubulin, PC tubulin was incorporated into microtubules after a lag time of 1 min. Electron microscopy revealed that the tubules formed from a mixture of PC and cycled tubulin were often defective. Incomplete closure of tubules resulting in regions that appear as flat ribbons of protofilaments were often seen. These data show that high molecular weight MAPs do not cycle with the fraction of tubulins obtained by temperature cycling. Since PC

tubulins do not spontaneously initiate assembly, there may be MAP fragments or lower molecular weight proteins required for initiation of polymerization.

Tidal rhythm and tissue organization in the neural gland of Ciona intestinalis: correlates with cellulose and fibronectin. JAMES W. LASH, MICHAEL OVADIA, CHARLES H. PARKER, AND CLIVE N. SVENDSEN.

Rarely have tidal rhythms been associated with specific tissue changes. It is more common that such rhythms are expressed as behavioral changes. In the ascidian *Ciona intestinalis* tidal rhythms have been found to be correlated with specific alterations in the neural ganglion and gland. At high tide the ganglion secretes PAS positive granules, but not at low tide. At low tide the gland is organized as a parenchyma and secretes an Alcian Blue positive substance. At high tide the neural gland undergoes a marked reorganization, and the gland cells now adhere to newly appearing PAS and Alcian Blue positive fibers. The function of the gland is poorly understood and the nature of the secretory products is not known. Ganglion/gland complexes isolated from the animal undergo complete transition from low tide phase to that of high tide, but not the reverse. In an attempt to mimic the gravitational forces thought responsible for these changes, intact animals were centrifuged for one tidal interval at $1.5 \times g$. This permitted the change from low tide phase to that of high tide, but maintained the high tide phase. Various enzymes were used to obtain evidence of the constituents of the ganglion/gland complex. Hyaluronidase digested the ganglion secretory granules. Cellulase removed most of the fibers in the gland at high tide phase. Using human and hamster fibronectin antiserum and fluorescein conjugated IgG, fluorescence was seen primarily in the neural gland, coating the fibers at high tide and generally distributed around the gland cells at low tide. Thus the extra-cellular matrix of the gland appears to contain cellulose and fibronectin.

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The growth and reproduction of selected species of meiofauna in selected natural microfloral assemblages. JOHN J. LEE AND MONICA J. LEE.

Previous research has shown that various species of meiofauna are highly selective in their feeding habits and that when incubated in gnotobiotic cultures with different species of algae and bacteria their growth and fecundity vary greatly. Sometimes two or more species are needed to satisfy the nutritional requirements of an animal. Enigmatically most of the meiofaunal species studied gnotobiotically are most fecund on species with low natural abundances ($\leq 3\%$). This raises the question of whether littoral benthic microbial assemblages are consumer controlled or whether they selectively control meiofaunal abundances and fecundity. Experiments carried out this summer in both the field and the laboratory provided more data on this important question.

Natural microfloral assemblages were gently sieved ($38 \mu\text{m}$) to remove all larger organisms and meiofauna. After the sieved mixtures were examined and the juveniles, nauplii, larvae, ciliates, etc. were picked out with the aid of small capillary pipettes, the mixtures were used as inocula for growth experiments. Selected meiofaunal species were inoculated into tissue culture flasks with the "natural mixtures" and incubated *in situ*. The tissue culture flasks had nylon filter ($3.0 \mu\text{m}$) covered windows cut into their surfaces which allowed free passage of sea water while retaining the algal assemblages. Vessels in assemblages without meiofauna and vessels incubated in the laboratory under constant environmental conditions served as controls.

Allogromia laticollaris, a foraminiferan, steadily increased in all the natural assemblages into which it was introduced. Populations of *Chromadorina germanica*, a nematode, reproduced vigorously and then crashed without recovery in one set of experiments and failed to reproduce in a mixture incubated later in the summer. *Rhabditis marina*, another nematode, failed to reproduce in either experiment. *Nitocra typica*, an harpacticoid copepod, reproduced after a lag of 3 weeks and maintained a high population for 7 weeks. Permanent preparations were made

of the diatom assemblages of the control and experimental vessels. Studies of these preparations are now in progress.

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The photosensitizing action of psoralens in dogfish ocular tissues. S. LERMAN, J. M. MEGAW, AND Y. TAKEI.

The photosensitizing action of 8-methoxypsoralen (8-MOP) has led to increasing concern regarding potential ocular complications in patients treated with this drug. Free 8-MOP can be demonstrated in the dogfish ocular lens and retina for 24 hr following an intraperitoneal injection of 1 mg of this drug. The 8-MOP can be detected in the lens by phosphorescence spectroscopy and labelled 8-MOP (25–50 μ Ci ^{3} H or 15 μ Ci ^{14} C) by autoradiography. When the fish is kept in the dark, the free 8-MOP diffuses out of these tissues after 24 hr. However, photic stimulation by exposure to G.E. BLB fluorescent light (mainly 365-nm radiation) for 1 to 15 days, results in the formation of photoproducts involving lens proteins and 8-MOP. The extracted and purified soluble protein fractions (after repeated TCA washing) had a significant activity (both for ^{3} H and ^{14} C) compared with background levels in proteins from controls kept in the dark. Autoradiographic studies were also positive in the tissues obtained from UV-exposed fish. These data correlate well with phosphorescence, EPR, and NMR experiments which demonstrate at least one photoproduct in the soluble lens protein fraction (*in vitro* and *in vivo*) following exposure to UV radiation longer than 320 nm. We have shown that the tryptophan 8-MOP photoreaction requires O_2 , in contrast with the cyclobutane photoproducts formed between 8-MOP and thymine, which are not O_2 dependent. Singlet O_2 plays a role in the 8-MOP-tryptophan photoreaction. Because the ocular lens is a completely encapsulated organ which grows throughout life and never sheds any of its cells, the photo-binding of 8-MOP and lens proteins (as well as with DNA) will result in the permanent retention of new photosensitizers within this organ. This could enhance the UV-induced changes already occurring in the normal aging lens and might even have a cataractogenic potential. Furthermore, the ocular lens normally acts as a UV filter, preventing UV radiation (longer than 300 nm) from damaging the retina. Thus the presence of 8-MOP in the retinas of aphakic patients could also prove hazardous.

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Possible mechanisms for inhibition of cellular activity by dyes with highly negative reduction potentials. DEBORAH LIPMAN AND SEYMOUR ZIGMAN.

Cyanine (methine) dyes with reduction potentials (E_R) more negative than -1.0 V have been found by us to inhibit mitosis in sea urchin embryos at 10^{-6} to 10^{-8} M. Chemically similar dyes with E_R values less than -1.0 V were ineffective. The motility of the dinoflagellate *Gymnodinium breve* was also eliminated by dyes with E_R values more negative than -1.0 V, but not with E_R values more positive (calomel electrode). Consideration of the possible mechanisms behind these varied effects and also the inhibitory effects on the synthesis of DNA and protein in sea urchin eggs by these dyes led to a hypothesis that the dyes with highly negative E_R 's interfere with cellular electron transport in oxidative metabolism and with energy production. The highly negative potentials (*i.e.*, greater than -1.22 V using the hydrogen electrode) of these dyes compared to those of NAD, FAD, and the cytochrome c half cells (all considerably more positive) would predict an action due to electronic saturation. Observations of the ability of sea urchin eggs (*Arbacia punctulata*) to divide, and measurements of their ATP content (measured by the firefly tail assay) after treatment with dyes having highly negative E_R 's indicated that no major reduction in ATP content resulted. These dyes may alter electron transport reactions involved in other phases of cellular oxidative metabolism, for example cytochrome c. Thus the inhibition of growth and reduction of activity of cells due to their exposure to dyes with highly negative E_R 's may not result from interference with energy metabolism, but could result from electronic saturation of the components of cellular respiration with other consequences.

(Dyes supplied by Eastman-Kodak Co.)

Presynaptic calcium current and postsynaptic response generated by a presynaptic action potential; a voltage clamp study in the squid giant synapse. R. LLINÁS, M. SUGIMORI, AND S. SIMON.

The present study was designed to characterize the presynaptic calcium current ($\text{Pre } I_{\text{Ca}}$) and its relation to postsynaptic potential (PSP) and current (PSI) during a presynaptic action potential. This was accomplished by driving the command amplifier of the presynaptic voltage clamp circuit with a prerecorded spike. In addition, the experiments would test whether the properties of the $\text{Pre } I_{\text{Ca}}$ predicted for a presynaptic spike by our model (Llinás *et al.*, 1976, *Proc. Nat. Acad. Sci.*, **73**: 2918) could be reproduced experimentally.

In the present study the waveform of a presynaptic action potential was recorded prior to the application of tetrodotoxin tetraethylammonium and 3-aminopyridine. Following addition of these agents, voltage clamp conditions at -70 mV holding potential were simultaneously set (via two independent circuits) to both pre- and postsynaptic elements in the junction in order to measure I_{Ca} and PSI (Llinás and Sugimori, 1978, *Biol. Bull.*, **155**: 454). The $\text{Pre } I_{\text{Ca}}$ measured during the spike-like presynaptic voltage clamp depolarization started near the peak of the presynaptic "spike", and its maximum amplitude had a sigmoidal dependence on the amplitude of the presynaptic voltage transient. Both peak and total postsynaptic current and peak PSP were linearly related to peak or total presynaptic I_{Ca} . This study confirmed the linear relationship between presynaptic calcium and postsynaptic response. Furthermore, the time course and magnitude for I_{Ca} coincided with those predicted by the model and with the conclusion that synaptic transmission is mainly produced by an "off" calcium current (*i.e.*, generated at the falling phase of the action potential).

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Stimulation of testosterone production by mouse Leydig cells with factors isolated from a microorganism and Ovalipes ocellatus. TAKESHI MAURO, AMY R. SEGAL, AND S. S. KOIDE.

During the course of determining choriogonadotropin (CG)-like substance in tissues of marine organisms, we have demonstrated that a trypsin-like protease present in the crab (*Ovalipes ocellatus*) stomach mimics CG in the radioimmunoassay (RIA) and the radioreceptor-assay (RRA) systems and is capable of stimulating rat ovarian adenylate cyclase activity *in vitro*.

In this communication evidence will be presented to show that a CG-like factor is produced by a microorganism. Extract of an acetone-dried preparation of a culture content of a microorganism tentatively named as "*Progenitor crytoides*" was prepared. The extract contained a CG-like factor as determined by RIA with the antiserum to hCG β -COOH-terminal peptide and RRA using bovine corpus luteum membranes. The CG-like factor was purified by chromatography on Sephadex G-100, Concanavalin A-Sepharose, and DEAE-Sephadex A-50. Interference by proteases in the extract was excluded. The bacterial factor was adsorbed on ConA-Sepharose, suggesting that it contains glucose or mannose moieties. Gel filtration on Sephadex G-100 demonstrated that the purified factor was eluted at the same position as standard hCG.

When the *in vivo* biological activity of the purified factor was determined by the uterine weight and the ovarian weight assays using immature female rats, the potency of the factor was 380 IU/mg and 900 IU/mg, respectively. Moreover, it stimulated testosterone production by mouse Leydig cells. Its biological potency was estimated to be equivalent to 3400 IU/mg. The CG-like factor purified from crab stomach induced a slight stimulation of testosterone production by mouse Leydig cells. The relative biological potency was equivalent to 0.3 mIU/mg. On the other hand, trypsin and chymotrypsin did not stimulate steroid formation.

The present findings suggest two alternative hypotheses for the presence and expression of mammalian gene(s) in bacteria and invertebrates. One is that the CG gene has its origin early in evolution, possibly as a protease. The other is that it is a consequence of a natural process of recombination.

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Studies on urn cell complexes of Sipunculus nudus (Linnaeus): influence of physiologic and pathologic mammalian sera on mucus secretion. LUIGI MASTROIANNI, JR., SANTO V. NICOSTA, ELLEN STREIBEL, AND GILBERT HAAS.

Serum may play an important role in both vertebrate and invertebrate mucus secretion. This possibility is intriguing since many factors which induce serum transudation, such as estrogen and immune complexes, are also associated with enhanced mucus release. In order to study the influence of different hormonal milieus and of altered immunologic states on the mucus-stimulating activity (MSA) of serum, urn cell complexes (UCC) of *Sipunculus nudus*, 50 to 100/exp., were incubated with unheated physiologic and pathologic sera. Sera were diluted 1:1 with filtered sea water (FSW). Mucous tails (mean μm tail length $\pm$ s.e.) were produced in response to male and female rabbit, mouse, and human sera obtained during endocrinologically different states. A significant ($P < 0.001$) enhancement of mucus release was induced by estrous mouse sera (67.22 ± 4.45 vs. 43.90 ± 3.06 at diestrus). Mucous tails of significantly greater length ($P < 0.001$) were also observed with sera obtained from estrous rabbits 12 to 15 hr after treatment with 5.0 mg of conjugated estrogen (45.97 ± 4.11 vs. 17.88 ± 1.87 , before) and 20 min after a single intravenous injection of 25 μg of estradiol-17 β (37.91 ± 3.75 vs. 14.00 ± 2.01 , before). The estrogen-enhanced MSA of serum was not altered by extraction with activated charcoal and, as indicated by dialysis, the active factor had a molecular weight greater than 13,000. Mucus release did not occur with FSW alone and with FSW containing 10 $\mu\text{g}/\mu\text{l}$ of estradiol-17 β or 10 to 20 mg/ml of bovine serum albumin. UCC also released mucous tails of significantly greater length ($P < 0.001$) when exposed to pathologic sera obtained from infertile or vasectomized men exhibiting circulating antisperm agglutinins (64.12 ± 1.57 vs. 16.82 ± 1.83 , normal serum). This secretory response was commensurate with agglutinin titers. These results indicate that mammalian sera may contain more than one factor capable of inducing augmented mucus secretion.

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Effects of extreme temperatures on protein synthesis in the toadfish. RITA W. MATHEWS AND AUDREY E. V. HASCHEMEYER.

The toadfish (*Opsanus tau*), a temperate species occurring along the eastern Atlantic from the Gulf of Maine to Cuba, tolerates a broad range of temperatures both in nature and in the laboratory. Studies of protein synthesis in liver *in vivo* have shown a moderate temperature dependency ($Q_{10}=2.5$) from 15° to 30° C. As temperature is decreased, Q_{10} increases dramatically; however, even at 4° C all steps of protein synthesis continue to operate slowly without apparent differential breakdown in function. Rates return to normal when fish are warmed.

At temperatures above 30° C polypeptide chain elongation showed a normal rate increase with temperature (Q_{10} about 2.5), reaching 6 amino acid residues per second at 37° C, as in mammalian liver. However, total incorporation of radioactive amino acids into protein, reflecting all steps in protein synthesis, declined precipitously above 32° C. Results were obtained as fractional incorporation rate (incorporation into protein divided by total radioactivity in liver and by incubation time). At 30° C the fractional incorporation rate for ^{14}C -leucine is 0.10 min^{-1} representing 10% turnover of the intracellular leucine pool per minute. In 1-min pulse experiments carried out *in vivo* immediately after the fish was warmed to the experimental temperature fractional incorporation was found to follow an Arrhenius relationship downward with Q_{10} about 1/40 up to the lethal limit of 39° C. Fish returned to 30° C after 5-min exposure to higher temperatures showed nearly complete recovery of synthetic rate up to an exposure temperature of 38° C. The results suggest a partially reversible lesion in the system involving initiation or recycling of ribosomal subunits.

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The size of the photosynthetic unit and its turnover time in various seaweeds.
D. MAUZERALL AND D. WITTENBERG.

The size of the photosynthetic unit (PSU), defined as the moles of chlorophyll (Chl) per mole of O_2 emitted in a single flash was determined following the classical experiment of Emerson and Arnold. Our previous measurements have shown that green seaweeds have about the same PSU size (2000 Chl/ O_2) as freshwater green algae and higher plants, but that the red seaweeds have a smaller size based on Chl a. We have extended these measurements to grasses and to other red and brown seaweeds. *Spartina alterniflora*, short: 2100 ± 300 , tall: 3600 ± 900 (A. Hogan); *Zostera marina*, 2200 (L. Mazzella); *Champia parvula*, 1500; *Ceramium rubrum*, 1100; *Gracilaria verrucosa*, high light 1600, low light 2100 (T. Kursar); *Fucus vesiculosus*, 1800; *Laminaria saccharina* 3300 (S. Schatz); *Sargassum filipendula*, 2000; *Sphacelotrichia divaricata*, 1900. Light absorption of the accessory pigments makes up for the missing Chl in the red seaweeds, but add to the absorption of the brown algae. A quantitative measure of pigment efficiency is the fraction of solar photons absorbed by the pigment. This is roughly equal for all the pigments over the full solar spectrum. This selection weakness may allow a more random play of evolution and account for the wide diversity in amount and kind of pigments observed. The turnover time is remarkably constant, near 0.5 msec, for all the species studied. This result shows a constancy in the evolution of photosynthesis that transcends the variable color and morphology of the benthic algae.

We thank Dr. J. Fiore for help in collecting and identifying the seaweeds, the named students in the program for data, and the Experimental Marine Botany Program and the NSF (grants PCM 74-11747 and 79-06638) for support.

Photosynthetic characteristics of Zostera marina L. (Eel grass). L. MAZZELLA,
D. MAUZERALL, H. LYMAN, AND R. S. ALBERTE.

The photosynthetic activity of *Zostera marina*, from a tidal bed in the Woods Hole area, was studied. It was found that the chlorophyll (Chl) content (mg Chl/g fresh weight and mg Chl/cm²), oxygen evolution rates [$\mu\text{mol } O_2/(\text{cm}^2 \cdot \text{min})$ and $\mu\text{mol } (O_2/(\text{mg Chl} \cdot \text{min}))$] and numbers of photosynthetic units (PSU) per unit leaf area varied in response to leaf age, light exposure and degree of epiphytization. These parameters were measured for different leaves from the youngest innermost leaf to the oldest outermost epiphytized leaves, and in different portions of the leaves from the youngest lowest portion to the oldest upper more epiphytized portion. The Chl content was lowest in the base of the youngest innermost leaves (0.002 mg Chl/cm²), while the highest concentration was found in the middle portion of old epiphytized leaves (1.56 mg Chl/cm²). The lowest photosynthetic activity [$2.5 \times 10^{-1} \mu\text{mol } O_2/(\text{mg Chl} \cdot \text{min})$] was found in the base of the youngest leaves while the highest activity was observed in the tip of old epiphytized leaves [$(10.5 \times 10^{-1} \mu\text{mol } O_2/(\text{mg Chl} \cdot \text{min}))$]. In general a gradient in Chl content and photosynthetic activity from the tip to the base of all leaves was found except in epiphytized old leaves from which the epiphytes were removed. The disappearance of this gradient from the latter leaves and the highest activity seen in the tip of old epiphytized leaves suggests that a significant portion of photosynthetic activity of older leaves is due to the presence of epiphytes. A gradient in Chl content and photosynthetic activity in the younger portion of all leaves from the outermost to the innermost leaves was also observed. The different maturity of the tissue and the different exposures to light intensity could explain this gradient in the young portion of different leaves. In conclusion, the photosynthetic activity of *Zostera* leaves is regulated by three factors: first, age of tissue; secondly, light intensity exposure; and thirdly, presence of epiphytes with all other parameters constant (i.e. temperature, depth). For the young part of all leaves the light intensity and the maturity of tissue play an important role. For the oldest epiphytized leaves, the presence of epiphytes is more important than the other factors in the dynamics of the photosynthetic activity and biomass production.

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Interaction of liposomes with dogfish lens capsules. J. M. MEGAW, S. LERMAN, AND Y. TAKEI.

Liposomes are microscopic vesicles composed of lipid bilayers. They have been used to deliver drugs and enzymes into tissues and cultured cells. Techniques employed to enhance the specificity of delivery have included antibody and lectin-mediated binding. We have investigated the possibility of binding concanavalin A (Con A) containing liposomes to the lens capsule of the dogfish both *in vitro* and *in vivo*. Liposomes were prepared by injection of ethanol solutions of phosphatidyl serine and phosphatidyl choline into PBS alone, or PBS containing either unlabeled or fluorescein labeled Con A. The vesicle size was increased by incubation in the presence of CaCl_2 followed by addition of EDTA. The liposomes were then washed by centrifugation and suspended in PBS. Whole dogfish lenses, some pre-incubated with unlabeled Con A, were incubated in the various liposome preparations and then washed in PBS. The capsules were examined by scanning electron microscopy (SEM) or by fluorescence microscopy (FM). For the *in vivo* studies, small volumes of aqueous humor were withdrawn from the eyes and were replaced with fluorescein-labeled liposomes in PBS. After 6, 23, or 29 hr, the eyes were removed and portions of the cornea, angle, iris, retina and lens were fixed for SEM or were examined by FM. SEM and FM demonstrated that liposomes containing Con A were bound to the lens capsule *in vitro*. Pre-incubation of lenses with unlabeled Con A resulted in decreased binding of Con A containing liposomes to the lens capsules. FM of eye tissues from animals injected with fluorescein-labeled Con A liposomes indicated binding of fluorescent material to the anterior lens capsule and to a lesser extent, to corneal endothelium. No fluorescence was noted in vitreous, retina or posterior lens capsule.

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*Nicotinamide deamidase activity in oocytes of *Spisula solidissima*.* AKIRA MOMII AND S. S. KOIDE.

We have demonstrated previously that 5 mM nicotinamide inhibits germinal vesicle breakdown (GVBD) in *Spisula* oocytes and that nicotinamide is rapidly converted to a metabolite by *Spisula* oocytes. These findings suggest that the active inhibitor of GVBD may not be nicotinamide itself, but a metabolite. The present study was performed to identify the metabolite, to determine the enzymatic system mediating the transformation, and to investigate the mechanism of GVBD inhibition induced by incubation with nicotinamide.

Packed oocytes (2.5 ml) were suspended in 9.5 ml artificial sea water (ASW) and incubated with 0.5 ml of ^{14}C -nicotinamide (25 μCi) at 22 to 23° C for 3 min. The final concentration of nicotinamide was 5 mM. The oocytes were washed with ASW three times and extracted with 2% HClO_4 . The extract was neutralized with 1 N KOH and centrifuged. The metabolite in the supernatant was identified as nicotinic acid by PEI-cellulose and DEAE-cellulose thin-layer chromatography (TLC). No nicotinamide was detected. Since it has been demonstrated previously that nicotinic acid has no influence on GVBD in *Spisula* oocytes, it is unlikely that this metabolite is the active agent. Since ammonia is a product of the transformation, the ability of ammonium bicarbonate to influence GVBD was tested. At 15 mM, ammonium bicarbonate blocked GVBD induced by insemination (45% GVBD compared to 95% for the control). Nicotinamide deamidase activity (EC 3.5.1.19) of *Spisula* oocytes was determined. Oocytes homogenate was incubated with ^{14}C -nicotinamide at 37° C for 15 min. The resulting nicotinic acid was quantified after separation on PEI-cellulose TLC developed with H_2O for 5 cm and with 1 N HCOOH for 10 cm. The deamidase activity is greater than 700 nmoles/(mg protein·hr) and does not change after fertilization. This value is far greater than that reported for mammalian tissues or the level found in *Arbacia* eggs. *Spisula* hepatopancreas has deamidase activity equivalent to that found in *Spisula* oocytes.

It can be concluded that *Spisula* oocytes possess an active deamidase system that rapidly transforms nicotinamide to nicotinic acid. The liberated ammonia may be responsible for the inhibition of GVBD in *Spisula* oocytes induced by nicotinamide.

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Differential solubilities of cytoskeletal proteins in squid axoplasm. JAMES R. MORRIS AND RAYMOND J. LASEK.

We have studied the forms which cytoskeletal proteins take under physiologic conditions using squid giant axon axoplasm. The axoplasm consists principally of cytoskeletal proteins (tubulin, neurofilament proteins (NFP), and actin). A cylinder of fresh axoplasm was extruded from the giant axon directly into a physiologic axoplasmic buffer (buffer P). Using SDS-PAGE, proteins diffusing into buffer P were compared to those remaining in axoplasm. After 24 hr at 20° C in buffer P, essentially all of the NFP (95%) remain in the axoplasm while 83% of the tubulin and 75% of the actin diffused into buffer P. Most of the other axoplasmic proteins appear only in buffer P. The axoplasm maintains its cylindrical morphology throughout the experiment. The mitochondria are retained in the axoplasm. Electron microscopy shows the presence of a network primarily composed of neurofilaments (NF). Microtubules are absent. This structure is referred to as the axoplasmic ghost.

Because all the NFP remained in the ghost and electron microscopy shows the ghost to be principally NF, we conclude that essentially all axonal NFP are normally polymerized in NF. A fraction of the tubulin and actin also remain attached to the ghost. This fraction must also exist as stable polymer. Most of the tubulin and actin diffused into buffer P. This diffusible component exists either as monomers or as a polymer which is soluble under physiologic conditions. We distinguish between monomeric and polymeric forms by analyzing the kinetics of protein diffusion into buffer P. This analysis shows that the diffusion of tubulin and actin includes a fraction which is slowed when compared to physico-chemical predictions. Thus, we have quantitatively analyzed three forms of cytoskeletal proteins in axoplasm: stable polymer, soluble polymer, and diffusible monomer. NFP differ from tubulin and actin in that NFP exist solely as stable polymer while tubulin and actin exist in all three forms in the axon.

Cytoskeletons in Labyrinthula slimeways. NORIO NAKATSUJI AND EUGENE BELL.

Electron microscopic observations have shown the existence of three kinds of filaments in *Labyrinthula* slimeways; many actin-like 6-nm filaments, rare short filaments that are thicker than 6-nm, and numerous short 2.3-nm filaments. The latter two kinds of filaments may be myosin aggregates and individual myosin molecules respectively. Decoration of the 6 nm filaments with myosin S-1 fragments was done after mild glycerination. They made clear arrow-head structures, showing that actin is contained in the filaments. Controls with 4 mM ATP showed no decoration. Polarity of parallel filaments was not always in the same direction. Decorated filaments were frequently observed to terminate in dense plaques associated with the slimeway membrane. Immuno-fluorescent staining, SDS-polyacrylamide gel electrophoresis and arrow-head decoration of filaments support the probability that actin and myosin occur in the slimeways.

Mg²⁺ and Ca²⁺-free sea water causes the slimeway to break into many beads. Thin sections of these beads show the attachment of 6-nm filaments to the slimeway membrane plaques clearly. Some thin sections of glycerinated slimeways suggest that 6-nm filaments are also attached to the outer surface of the unit membrane of intra-slimeway vesicles. Thus, for a model mechanism of cell translocation, we propose that actin filaments are attached to the inner surface of the slimeway membrane and to the outer surface of the membrane that is investing the spindle cells, to permit myosin-mediated sliding between these filaments.

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Marginal band systems in blood cells of marine species: visualization by indirect immunofluorescence. IRIS NEMHAUSER, WILLIAM D. COHEN, AMY MILSTED, AND ROBERT D. GOLDMAN.

Marginal bands (MBs) and associated paired centriole-like structures ("perioles"; possible MB organizing centers) were observed in Triton-lysed red cells of the mollusc *Anadara*

transversa, using indirect FITC immunofluorescence with anti-tubulin. Only MBs and the associated perioles fluoresced, with the disposition of the latter matching that observed in the same lysed cells in phase contrast. In some cases MB fluorescence was so great as to obscure that of the perioles, though they were clearly visible in phase contrast. Similar results were obtained with preparations of lysed dogfish erythrocytes (*Mustelus canis*), except that MB-associated, paired fluorescing structures were observed in a minority of cells. It is possible that they are present only in immature cells or obscured in most cells by the MBs or other structures. The results suggest that centriole-like structures may be associated with the MBs of diverse species, and that a careful search for them is warranted.

Anti-tubulin binding was also employed to follow structural alterations occurring in MBs of lobster coelomocytes (*Homarus americanus*) as they undergo changes in morphology while spreading on glass substrata (at room temperature, approximately 21° C). At $t = 0$ (no spreading) the cells are flattened and somewhat elliptical, with virtually all of their microtubules contained within intact circular or elliptical MBs. After spreading for 5 min ($t = 5$ min), most MBs are still recognizable but show signs of disorganization, with figure-8 forms prevalent. At $t = 10$ min MBs are no longer recognizable as such, with bundles of microtubules splayed in various directions. At $t = 20$ min the microtubule network has disappeared, leaving only scattered points of fluorescence visible in some cells. This is in contrast to other cell types such as those of cultured cell lines, which reorganize microtubule networks as they spread. Perhaps, in this instance, the cells are engaged in a process of self-destruction related to a clotting role *in vivo*.

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Ultrastructure of urn cell complexes of Sipunculus nudus (Linnaeus) before and after serum-induced mucus release. SANTO V. NICOSIA AND JANICE SEWINSKI.

This report analyzes the cytology of urn cell complexes (UCC), a currently used invertebrate model of humorally-regulated mucus secretion. UCC are 60–80 μm in size and are composed of one vesicle cell (VC) and one basal cell (BC) marginally joined by prominent desmosomes. VC's are thin-walled structures whose cytoplasm contains few mitochondria and scattered, sudanophilic and osmiophilic, lipid droplets. These cells surround a vesicular cavity which contains mucus-like, microfibrillar material and is continuous with the extracellular milieu through the BC-delimited opening of UCC. There is a distinct polarity in cilia distribution and secretory activity in the BC. Rows of 5 to 10 μm long cilia are present, amidst microvilli, exclusively in the outer lining of BC. These cilia are anchored by 3 to 5 μm long, curving rootlets which exhibit a periodicity of 65 nm and may have, together with desmosomes, a role in UCC plasticity during the mechanical stresses of mucus release, debris sweeping, and forward propulsion. In addition to numerous ribosomes, scattered mitochondria, and few lipid droplets, BC contain approximately 8 to 10 $\times 10^3$ secretory-like granules which are uniformly electron-dense and lack a distinct limiting membrane in glutaraldehyde-osmium fixed UCC. However, they display cytochemical characteristics of mucous granules (diastase resistance, periodic acid-Schiff positivity, metachromasia, and immunoperoxidase-detectable lysozyme activity). The number of these granules is also drastically reduced after serum-induced mucus release. Typical exocytotic discharge of secretory material into the vesicular cavity of UCC is rare. More often these granules are found around or within intracellular canaliculi or sacs which extend up to the inner lining of BC where mucus release takes place. BC also contain 4 to 5 circular bundles of 14 to 17-nm wide microfilaments whose integrity, along with the UCC ability to release mucus, is disrupted by cytochalasin B. This study suggests that mucous tails are formed on the inner aspect of BC by a microfilament-mediated extrusion of mucus from storage reservoirs (intracellular canaliculi and/or the vesicular cavity) of UCC.

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Particle size selectivity in Modiolus demissus and Balanus balanoides. DEANNA H. OLSON.

In great Sippiswissett Salt Marsh, Falmouth, Massachusetts, *Balanus balanoides* settles on *Modiolus demissus*, as *M. demissus* shells comprise most of the available hard substrate in the marsh system. Particle-size selectivity of these filter feeders was studied and related to possible food competition which may limit growth in areas of the marsh. Each species was allowed to filter natural sea water in a standing culture system. The water contained a decreasing logarithmic progression of particles with increasing size. Particle concentration was determined (Coulter counter: 5-49 μ m size range) before introduction of the species to the system, and after 2 and 4 hr of filtration (7-11 replications).

Mean percent removal of seven particle size classes, within the range studied, was calculated for *M. demissus* (valve length range: 71-74 mm) and for a number of individuals of *B. balanoides* which corresponded to the average observed on marsh mussels. For both species, percent removal of particles increased positively with increasing particle size. Within 2 hr, the removal of particles in each size class by *M. demissus* ranged from 68 to 94% and by *B. balanoides* from 10 to 90%. After four hr, *M. demissus* and *B. balanoides* filter 93 to 99% and 19 to 95% of available particles, respectively. Particle-removal spectra for *M. demissus* suggested generalized filtration while *B. balanoides* selectively removed larger particles. Equal biomasses of these species (obtained by adjusting barnacle numbers) yielded particle removals similar to the above. Therefore competition between these filter feeders occurs only for the larger-sized particles studied.

Ammonification and nitrification potentials of soils from a northern hardwood forest and a pine plantation. K. C. PARSONS AND J. M. MELILLO.

In a laboratory study, rates of ammonification and nitrification were measured for organic and mineral soils from a northern mixed hardwood forest and a pine plantation. Two-normal KCl extractions of organic soil samples from both stands yielded no nitrite-nitrate after nine weeks of incubation, although more than 250 μ g of ammonium-nitrogen per gram soil (dry weight) was extracted from the same samples. Only mineral soil samples from the hardwoods produced significant, albeit low, levels of nitrite-nitrate (8.0 μ g $\text{NO}_2 + \text{NO}_3\text{-N/g}$ soil from the upper 15 cm mineral soil, 2.5 μ g $\text{NO}_2 + \text{NO}_3\text{-N/g}$ soil from 15 to 30 cm mineral soil horizon). Levels of ammonium extracted from mineral soils from the hardwood stand ranged from 2.5 to 8.0 μ g $\text{NH}_4\text{-N/g}$ soil. Mineral soils from the pine plantation produced 1.5 to 5.0 μ g $\text{NH}_4\text{-N/g}$ soil.

Other soil samples were leached with distilled water periodically for thirteen weeks to give rates of nitrification. Levels of nitrite-nitrate were significantly greater than ammonium in samples from the upper 15 cm of mineral soil from the hardwoods. No nitrite-nitrate was leached from the organic soils from either stand, and none was produced by the mineral soils from the pine plantation. Ammonification was evident in all soil samples, but especially prominent in the organic horizons. When soils were fertilized with phosphorus, nitrification rates remained essentially zero. Ammonification in amended samples was not significantly different from controls in any soil type from either stand. Both systems are concluded to be ammonium-dominated. These experiments show nitrifiers not to be phosphorus-limited. Directions for further research are suggested.

The histochemistry of muscle fiber types in the regenerating claws of the lobster, Homarus americanus. NATALIE G. PASCOE.

Lobsters regrow autotomized claws initially as soft limb buds, which upon the subsequent molt become hard-shelled, functional claws. Histochemical techniques developed by Mark Ogonowski and Fred Lang for the characterization of lobster muscle in respect to fast and slow properties were used to examine the state of differentiation of regenerating opener and closer muscles in claw limb buds from adult animals. Early in the development of a bud, the muscles stain darkly for NADH diaphorase, an indicator of oxidative capacity (fatigue

resistance). However, initially, myofibrillar ATPase, an enzyme that regulates speed of contraction, stains faintly. The staining patterns in mature buds are similar to those of the adult cutter and crusher claws. In the closer muscle of the mature bud alternate staining of sequential sections reveals that fibers that stain intensely for myofibrillar ATPase stain lightly for NADH diaphorase. Conversely, fibers that stain darkly for NADH diaphorase show little myofibrillar ATPase activity. Some single fibers of intermediate staining intensity are found, and they stain in an intermediate extent for both enzymes. High specific activity myofibrillar ATPase seems to exclude the property of high oxidative capacity, or vice versa.

Opener muscles show regional differentiation in respect to NADH diaphorase; however, they do not have high specific activity myofibrillar ATPase in areas of low NADH diaphorase. They may lack the potential for this.

The diurnal response of the gray seal, Halichoerus grypus, to tide and insolation during the month of April at Muskeget Shoals, Nantucket Sound, Massachusetts, USA. DAVID PATON.

The integument of the gray seal undergoes a once-yearly molt during April in these latitudes. This metabolic activity increases the nutritional requirements of the animal because it remains on land. Heating of the skin by insolation augments this metabolic stress to the advantage of the animal. If the seal can spend the time of the molt cycle in air (a fluid of a lower rate of thermal conductance than water), it can sustain the molt in less time, thence saving energy that would normally be used in finding and processing food at sea. The gray seal has been observed to respond directly to the period of the diurnal tide cycle outside of the breeding and molting periods of the year. This behavior is supposed to be associated with feeding at sea.

During 5 days in April, a pod of gray seals was observed at Nantucket Sound. White light reaching the area was measured with a hand held Vivitar light meter, M90, and compared with a pyronometer, Epply 6-90, located at Woods Hole. Cloud cover, wind, sea and air temperature were also noted. Tide movement was observed on the study site and compared with published tide tables for the effects of wind fetch and current. Two gales swept the area during this time. The animals were not observed at night. A control animal, being offered its usual diet, was reported to have molted in an aquarium on Cape Cod isolated from tides. Climate diagrams were plotted for four size-color classes of model gray seals. The gray seals were observed to respond to insolation of white light rather than the period of the tide during the days of April 12th to 17th, 1979. Thermal regulatory behavior was observed. Further investigations into the bioenergetic bounding effect of this habitat on the gray seal will be continued.

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Distribution of gametophytes and sporophytes of Chondrus crispus in the vicinity of Woods Hole. NANCY PENNCAVAGE, SCOTT SCHATZ, ESTHER MCCANDLESS, AND JAMES FIORE.

The red alga *Chondrus crispus* of the North Atlantic coast derives economic importance from its high content of kappa and lambda carrageenans, sulfated galactans which differ in degree and position of sulfation and in the presence of an anhydro-ring in the former. Kappa carrageenan gels in the presence of K^+ ; lambda carrageenan solutions are viscous but do not gel. The variability in proportions of the two polysaccharides in carrageenan preparations was explained recently by the discovery by one of us that kappa carrageenan is produced only by gametophytes, lambda carrageenan only by sporophytes of *C. crispus* and certain other red algae. In these species, qualitative analysis for anhydrogalactose can therefore be used to determine the phase of nonsporulating plants. Northern collections of *C. crispus* have demonstrated that the percentage of sporophytes in populations increased with depth, but no information is available on relative proportions or distribution of the phases here near the southern limits of the species.

Between June 21 and August 18 we made 11 collections from 8 different Massachusetts sites: Black Rock, Cuttyhunk jetty, NMFS jetty, MBL beach, Menemsha jetty, Nahant, Nobska, and West Falmouth jetty. At NMFS jetty, Menemsha and Nobska, collections were made at several depths. A number of randomly selected plants from each collection (30 to 40) were analyzed for anhydrogalactose. Usually the proportion of sporophytes at mean low water (MLW) level was very low, approximately 5% of plants. At Menemsha this increased to 40% at -12 feet (below MLW), a distribution similar to that seen farther north. However, on both Nobska jetty and NMFS jetty the proportion of sporophytes was higher at MLW (22-28%) and reduced with greater depth (5% at -7 ft and 11% at -9 ft, respectively.) Explanation of these observations is not yet apparent. (Support of the Experimental Marine Botany program and NSF grant PCM-7906638 is acknowledged.)

Spectroscopic and chemical studies of protein-methylglyoxal complexes. RONALD PETHIG AND JANE A. McLAUGHLIN.

Szent-Györgyi has drawn attention (1979, *Biol. Bull.*, 157: 398) to the rôle of methylglyoxal (MG) in forming charge-transfer reactions with protein molecules. Such a concept has received strong experimental support from the studies of dielectric and electron spin resonance properties of protein-MG complexes (see Bone, 1979, *Biol. Bull.*, 157: 358; Gascoyne, 1979, 157: 369). Our purpose has been to investigate the basic chemical reaction responsible for such a charge-transfer interaction. A neutral aqueous mixture of bovine serum albumin (BSA), casein or lysozyme with twice distilled MG assumes a yellow color having an absorption peak at 330 nm and a "shoulder" at around 350 nm. Addition of NaBH₄ removes these absorption peaks. If the reaction is performed at pH4 the initial solution remains colorless. Adjustment of a neutral protein-MG aqueous mixture to pH3 modifies the absorption to give a new peak at 340 nm and no "shoulder" at 350 nm, and this effect is reversible on readjustment to pH7. The ϵ -amino groups of the lysine side-chains of BSA and casein have been reductively dimethylated and after this treatment the proteins no longer react with MG to form a yellow color. The blocking of the arginine side-chains of BSA with cyclohexanedione has produced no observable effect on the color reaction with MG. Studies have also been made using methanol-water mixtures.

We consider that these studies show that the relevant reaction for our studies involves the formation of a Schiff base between methylglyoxal and the protein lysine side-chains. Theoretical calculations by others show that such a Schiff base provides a good electron acceptor for charge-transfer interactions with neighboring peptide units. This forms a strong theoretical basis for the electronic desaturation concepts described by Szent-Györgyi as being relevant to the "living-state."

L-leucine transport by toadfish liver studied by the Oldendorf method in vivo. ROGER PERSELL AND AUDREY E. V. HASCHEMEYER.

A technique originally developed by Oldendorf for the study of transport in mammalian brain was extended by Pardridge (1977, *Am. J. Physiol.*, 232: E492-E496) to amino acid transport in rat liver. A pulse injection of ¹⁴C-amino acid and ³HOH is given via the hepatic portal vein, and retention of amino acid relative to water is evaluated as the Liver Uptake Index (LUI).

We have examined L-leucine uptake by toadfish liver *in vivo* at two temperatures by this technique. Hepatic clearance of ³HOH was followed as a function of time and the LUI for L-leucine determined. Maximal ³HOH extraction was about 80% of dose, similar to the value found in rat, but washout was much slower. In the time range up to 1.5 min, t_1 was 2.5 min at 20° C and 2.8 min at 10° C, compared to 0.8 min for rat. This represents a three-fold difference in rate of portal blood flow between the two species. LUI for leucine in toadfish was 0.7 to 0.8 for the first 4 min after injection and then increased as ³HOH continued its efflux from the liver. At $t=10$ min, LUI based on free (acid-soluble) radioactivity was 2.0 at 20° C. If protein-bound radioactivity is included in the LUI, following the method of Pardridge,

LUI is as high as 5. The latter value, however, reflects protein synthesis more than transport activity.

Previous studies in toadfish liver have shown concentration of L-leucine relative to an extracellular space marker, ^3H -inulin. This procedure has been used to study saturation and competition for the carrier-mediated transport process. The Oldendorf technique appears to be less useful in the fish because of the slowness of blood flow. Thus, the kinetics of liver amino acid transport when analyzed relative to HOH at the critical early times after injection are obscured by the slow water efflux.

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The relative importance of bacterial and fungal biomass and Spartina organic matter in the nutrition of two species of salt marsh amphipods. NEAL W. PHILLIPS.

Several recent studies question the generalization that microbes are the major food source for detritus-feeders. I examined the relative importance of microbial and non-microbial biomass in the nutrition of two species of marsh amphipods, *Orchestia grillus* and *O. spartinophila*. Assimilation efficiencies for total organic matter and for bacteria and fungi were estimated by an ash-tracer method which requires measurements of the ash fraction and of bacterial and fungal densities in food (dead leaves of *Spartina alterniflora*) and feces.

Specimens of *O. spartinophila* were fed submersed in chambers designed to minimize microbial growth on fecal pellets. Specimens of *O. grillus* were fed in moist fingerbowls from which fecal pellets were rinsed every 4 hr. After 12 hr of feeding, fecal pellets and uneaten food were homogenized and preserved for bacterial and fungal counts. Bacteria were enumerated by the acridine-orange direct count method; fungal hyphae were measured microscopically on filtered subsamples incubated with water-soluble aniline blue, a fluorescent stain. Fecal pellets from food of known ash content were dried, weighed, combusted (500°C , for 3 hr) and reweighed to obtain ash fractions.

Orchestia spartinophila assimilated 64 and 23% of total organics in two experiments. Assimilation efficiencies (5 replicates) for bacteria ranged from 23 to 75% and for fungi from 0 to 60%. The animals did not scrape fungi from leaf surfaces. *Orchestia grillus* apparently assimilated 39% of total organics and 60 to 70% of bacteria, but fungal assimilation efficiencies were highly negative. This suggests that the animals selectively ingest fungi, and that the calculated efficiency for total organics is incorrect. Bacteria averaged 0.11% of litter dry weight, and fungi 0.24%. Microbial biomass may represent a small fraction of total assimilable organic matter.

Detection of a synexin-like soluble factor in anglerfish islet tissue that aggregates islet secretory granules in the presence of small amounts of calcium. HARVEY B. POLLARD, BRYAN D. NOE, AND G. ERIC BAUER.

Exocytosis is a common mechanism for the secretion of hormones and transmitters that are transiently sequestered in secretory granules, and depends in many cell types on an initial rise in the intracellular calcium concentration. Elevation in calcium appears to cause secretory granules to become closely associated with either plasma membranes or, frequently, with the membranes of other secretory granules that have already secreted but are still attached to the plasma membrane. This calcium-dependent membrane interaction may be mediated by a new protein called synexin. Synexin was recently discovered in chromaffin cells where it caused chromaffin granules to aggregate with each other and with plasma membranes only in the presence of calcium. Synexin activity can be quantitated simply by following the increase in turbidity of a granule suspension at $\lambda 420\text{ nm}$ that accompanies aggregation, and we have utilized this approach to search for synexin activity in crude extracts of Anglerfish (*Lophius americanus*) islet tissue. We found that the post-mitochondrial supernatant fraction contained a potent islet granule aggregating activity which could also be observed directly by phase microscopy. Further analysis showed that the reaction was strictly dependent upon calcium,

since neither barium, strontium nor magnesium (all 2 mM) could either substitute for or inhibit calcium action (1.2 μ M). A careful titration of the calcium concentration dependence with an EGTA buffer revealed that the K (1/2) for granule aggregation was *ca.* 0.04 μ M, with a Hill coefficient of *ca.* 3.5. Furthermore, the activity was stable to both dialysis and boiling, the latter observation clearly distinguishing Anglerfish islet factor from bovine synexin. Nonetheless, the general similarities of the reactions catalyzed by both factors lead us to conclude that secretion of some hormones found in islet tissue may prove to be mediated by a synexin-like activity similar in some ways to the protein previously characterized in chromaffin tissue.

Succession of five common salt marsh detritivores on Spartina alterniflora detritus of decreasing particle size and increasing age. CATHERINE N. POURREAU.

Detritus from the common cord grass *Spartina alterniflora* in little Sippewissett salt marsh, Falmouth, Massachusetts, is the primary source of food for five invertebrate detritivores (high marsh: *Philoscia vittata* (isopod), *Orchestia grillus* (amphipod); low marsh: *Gammarus palustris* (amphipod), *Melampus bidentatus* and *Littorina littorea* (gastropods)). Feeding preferences of these detritivores for different physical and chemical states of decomposition of *S. alterniflora* leaves were studied by observing feeding activity in a quartered petri dish. Experiments (replicated three times) consisted of observations of animal positions in the dish every 10 min. *Orchestia grillus* and *P. vittata* proved photosensitive, hence were tested in the dark. *Melampus bidentatus* fed only in the dark.

Selection for specific detrital sizes and age classes were detected in all species. Coarse particles (0.6 cm leaf fragments) were selected by *M. bidentatus* and *L. littorea*, fine particles (0.6 mm) by *G. palustris*, and very fine (0.2 mm) by *P. vittata* and *O. grillus*. Conversely, 0- to 1-month-old detritus was preferred by *P. vittata*, 2- to 5-month-old detritus by *M. bidentatus*, 6- to 9-month-old detritus by *G. palustris*, and 9 months or older detritus by *L. littorea* and *O. grillus*. Relative importance of detrital particle size versus age for detritivores was tested by offering detritus of preferred age of an unpreferred particle size, and *vice versa*. *Philoscia vittata* and *O. grillus* responded more strongly to detrital age; *G. palustris*, *M. bidentatus*, and *L. littorea* to particle size. Consideration of these results and habitats of these detritivores suggests that the detrital resource is partitioned and does not overlap.

Transmembrane movements of sulfur compounds in the squid giant axon. R. D. PRUSCH AND F. C. G. HOSKIN.

The intracellular ionic content, including anions, is closely regulated by a variety of diffusional and active processes. Isethionate, 2-hydroxyethanesulfonate, accounts for nearly half of the total anion balance in the squid giant axon. Both sulfide and cysteine (specifically the sulfur of cysteine) are involved in the biosynthesis of isethionate and hence in the maintenance of the anion balance. Thus measurements of transmembrane movements of both cysteine and sulfide are important to an understanding of the anion balance in the squid axon.

When isolated squid axons were equilibrated with ^{35} S-cysteine (10^{-6} M cysteine externally) dissolved in sea water (1 mM HEPES, 10^{-4} M DTT, pH 7) a steady state intracellular concentration of 18 μ M was reached in 2 to 3 hr. Cyanide in the bathing medium (1 mM) reduced the internal cysteine concentration to 8 μ M in the same time. When isolated axons were injected with ^{35} S-cysteine, an efflux rate constant of about $1.15 \times 10^{-3} \text{ min}^{-1}$ was attained after a 1 to 1½ hr equilibration period. A reversible increase in cysteine efflux was brought about by 1 mM CN or 1 mM cysteine in the external medium. When the experiment was performed with 10^{-5} M Na_2S , the internal concentration of sulfide after 5 hr was 10 μ M. Cyanide (1 mM) reduced this to 6 μ M. The rate constant for ^{35} S-sulfide efflux was found to be $2 \times 10^{-3} \text{ min}^{-1}$ 2 hr after injection of the isotope. This was reversibly increased to $3 \times 10^{-3} \text{ min}^{-1}$ by 1 mM sulfide in the external medium and to $2.7 \times 10^{-3} \text{ min}^{-1}$ in the presence of 1 mM CN.

In summary both cysteine and sulfide uptake are reduced by CN, and the efflux of both are increased by CN. More importantly, cysteine efflux is stimulated by external cysteine, and sulfide efflux is stimulated by external sulfide. These observations indicate that both cysteine and sulfide are transported by carrier-mediated processes.

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Chemoreception in Homarus americanus: responses of primary receptors to secondary plant compounds. PAMELA REILLY, CHARLES DERBY, AND JELLE ATEMA.

Secondary plant compounds are known feeding inhibitors in terrestrial systems; their role in the marine environment, however, has only recently been examined. The major secondary compounds of terrestrial plants have generally not been found in marine plants; other compounds, such as phenolics, seem to be more important. The chemosensory basis of detection of these compounds by marine consumers is unknown. This study is a neurophysiological analysis of the sensitivity of lobster chemoreceptors to terrestrial and marine secondary plant compounds, including a comparison of responses of two chemoreceptive appendages: antennules and walking legs.

Extracellular responses from nerves of excised appendages were recorded while chemical stimuli were injected into sea water flowing over the chemoreceptive region of the appendage. Secondary plant compounds tested include those known in terrestrial environments (atropine sulfate, sinigrin, caffeine, salicin, amygdalin, morin, quinine sulfate, heliotropine) and marine environments (ferulic acid, *p*-coumaric acid, gallotannin, phloroglucinol, bromoform, diiodomethane). Mean responses (average number of spikes per trial) for these compounds were compared to those of two known excitatory stimuli: L-glutamate of equimolar concentration (10^{-6} M) and a standard mussel extract. Mean responses to L-glutamate were identical for leg and antennular chemoreceptors: 22% of the mussel extract response. The mean responses to secondary plant compounds were generally low, ranging from 0 to 60% of the L-glutamate mean response. The mean responses to these compounds were almost always higher for leg than for antennular chemoreceptors; this is due, at least in part, to the larger number of responding neurons in the legs. This implies that there is a larger population of receptors sensitive to secondary plant compounds in legs. This greater sensitivity of legs to secondary plant compounds indicates a functional difference between antennular and leg chemoreceptors, the latter being more important in the ultimate acceptance or rejection of food. This difference parallels that of smell and taste and is another argument in the functional separation of two chemoreceptor systems in aquatic vertebrates and arthropods.

Image intensification as a tool in low level fluorescence studies of living cells. GEO. T. REYNOLDS.

Wolniak *et al.* (1979, *Biol. Bull.*, 157: 402) describe results obtained using a high gain image intensification system for fluorescence studies of *Haemaphysalis* cells in a situation where the excitation intensity level required by conventional recording techniques destroys the cell development under study. The high gain system is an area detector consisting of a 4-stage intensifier capable of photon gains up to 10^6 , and the output phosphor is viewed with a plumbicon vidicon, SIT vidicon, or conventional film camera. In this work the excitation level was reduced by several orders of magnitude by means of filters. This, and even further reduction made possible by the high gain system, is also an advantage in studies in which: first, the high level excitation normally required "bleaches" the fluorescent probe; secondly, fluorescence tagging is limited in order to avoid interfering with normal cell processes; or thirdly, receptors for the probe are sparse. In observations in which background fluorescence is a problem, it is possible to utilize a very narrow band width excitation to enhance the signal to background ratio. With the low excitation levels possible using high gain image intensification, the required bandwidths can be achieved using a suitable monochromator rather than with the relatively complex and expensive tunable dye lasers required for high intensity excitation.

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The use of Limulus amoebocyte lysate (LAL) for the removal of lipopolysaccharide from biological reagents. FREDERICK R. RICKLES AND JACK LEVIN.

We have previously reported that many reagents are contaminated by lipopolysaccharide (LPS) (or endotoxin). Contamination of materials by LPS may interfere with the interpretation of results in various biological systems since LPS can mimic the action of many reagents and inhibit the action of others. For example, concanavalin A (con A), which is a potent mitogen for mammalian mononuclear cells, is frequently contaminated by LPS, thus limiting its usefulness as a specific B-cell lectin. We report here the results of separation experiments designed to remove LPS from con A by differential absorption with a lysate made from the amoebocytes of *Limulus polyphemus*. Con A was radiolabeled with ^{125}I and then mixed 1:1 (V/V) with LAL. After gelation and high speed centrifugation only $33\% \pm 9.8$ (s.e.) of the radioactivity could be recovered from the supernatant. If, however, the lectin was pre-incubated with the disaccharide alpha-methyl-D-mannoside, $80.5\% \pm 10.6$ of the radioactivity was in the supernatant. Four different preparations of con A have undergone purification by this method with complete removal of LPS. Each preparation has remained fully mitogenic when tested with human mononuclear cells (mean maximum DNA synthetic response = 7259 ± 2355 cpm before LAL absorption: 7477 ± 3410 cpm after LAL absorption). Similar results have been obtained with other protein antigens and lectins. Therefore, LAL can be used to remove LPS from a variety of proteins without loss of activity. In this way reagents can be prepared which have greater specificity and the pitfalls of LPS contamination can be avoided.

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Intracellular pH regulation in squid giant axons. J. M. RUSSELL AND W. F. BORON.

Squid axons respond to internal acid loads by returning intracellular pH (pH_i) toward normal (~ 7.3), in a process ("acid extrusion") which requires internal Cl^- and external Na^+ and HCO_3^- . Thomas (1977, *J. Physiol.*, 273: 317-338) has proposed that external Na^+ and HCO_3^- exchange for internal H^+ and Cl^- . Alternatively, Na^+ may combine with CO_3^{2-} to form the NaCO_3^- ion pair which enters in exchange for Cl^- . Both models predict a stoichiometry of 1 Na^+ :1 Cl^- :2 H^+ neutralized intracellularly. We have now used ^{22}Na , ^{36}Cl , and pH microelectrodes to measure the stoichiometry on internally dialyzed squid giant axons (inside: $\text{pH} = 6.5$, $[\text{Cl}^-] = 150 \text{ mM}$; outside: $\text{pH} = 8.0$, $[\text{Na}^+] = 425$, $[\text{HCO}_3^-] = 12$, $\text{TTX} = 10^{-7}$, ouabain 10^{-6}). The HCO_3^- -dependent Na^+ influx was 3.1 ± 0.5 (s.e.) ($n = 5$) $\text{pmol}/(\text{cm}^2\text{-sec})$. Na^+ efflux and Cl^- influx were -0.1 ± 0.3 (3) and -0.2 ± 0.3 (3), respectively. Cl^- efflux, taken from 1976 experiments (similar conditions), was 3.9. The acid extrusion rate, calculated from the pH_i recovery rate after halting dialysis, was 8.8 ± 0.8 (10). These data are thus consistent with predicted 1:1:2 stoichiometry. In addition, we studied the dependence of acid extrusion rate on $[\text{HCO}_3^-]_o$ and on $[\text{Na}^+]_o$. Values for apparent K_m and V_{max} were $2.3 \pm 0.2 \text{ mM}$ and $1.1 \pm 0.05 \text{ pmol}/(\text{cm}^2\text{-sec})$, respectively, for HCO_3^- , and $77 \pm 13 \text{ mM}$ and 1.1 ± 0.6 for Na^+ . When these acid extrusion rates were plotted as a function of $[\text{NaCO}_3^-]_o$, the data from the HCO_3^- and the Na^+ experiments fell on the same curve, with best fit values of $74 \pm 3 \text{ }\mu\text{M}$ for K_m , and 1.1 ± 0.2 for V_{max} . This is consistent with the ion pair model, but does not rule out the Thomas model. Acid extrusion was reversibly inhibited 80 to 85% by 1 mM furosemide and by 1 mM 4,4'-dinitro-2,2'-stibenedisulfonic acid.

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A comparative study on Laminaria saccharina (Phaeophyta) infected by Phycomelaina laminariae (Ascomycotina). SCOTT SCHATZ, DAVID MAUZERALL, AND JAMES FIORE.

A comparative study on the brown alga, *Laminaria saccharina* infected by the ascomycete, *Phycomelaina laminariae* is presented. Light microscopy reveals that fungal hyphae grow

intercellularly in the epidermis and outer cortex of host stipe tissue. Affected host cells become elongated and distended prior to cell-wall breakdown. Host plant susceptibility appears to be in part a function of the aging process with sporophytes younger than one year old displaying complete resistance to infection. The percentage of the population infected increased from 14.8 in May to 42 in August. Growth rates of infected plants was much less than that measured in healthy plants. Examination of the photosynthetic capabilities of blade tissue from healthy and infected plants shows that the photosynthetic unit sizes (psu) are similar. The psu in healthy plants is 3400 ± 100 while in infected plants the psu is 3000. However, the rate of oxygen evolution in saturating light was much greater in healthy plants than in infected plants. Significant differences were also found when transmitted light was reduced by 56, 74, and 90%. These data suggest that the dark reactions of photosynthesis are inhibited and not the light reactions. The following hypotheses are presented as possible mechanisms by which growth and photosynthesis are inhibited in infected host plants: first, the fungus serves as a sink, drawing away metabolites essential for the dark reactions of photosynthesis; secondly, the transport of photosynthetic products into the stipe is reduced resulting in a feedback inhibition of the dark reactions in the blade; thirdly, a mycotoxin produced by the fungus is transported from the stipe into the blade; and fourthly, these phenomena are simply a result of natural senescence processes typified by reduced metabolic activity thereby predisposing the plant to fungal infestation.

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Direct effect of LHRH on testicular steroidogenesis in Rana pipiens. S. J. SEGAL
AND C. A. ADEJUWON.

The LHRH (luteinizing-hormone-releasing-hormone) decapeptide has been identified in hypothalami of mammals, birds, and amphibia. For all vertebrate species studied, the role of releasing hormone in controlling LH secretion by the pituitary can be demonstrated. Recently, attention has been directed toward possible extra-pituitary effects of the decapeptide in order to explain paradoxical inhibitory action of LHRH or its analogues on gonadal function in mammals.

The direct effect of synthetic LHRH and one of its analogues (d-ser(bu)⁴desgly¹⁰ LHRH ethylamide) on steroidogenesis by the testis of *Rana pipiens* has been investigated. The study includes the influence of the polypeptides on the well-known stimulatory action of hCG on the frog testis. Individual testes of specimens of *R. pipiens* (in breeding season and producing abundant spermatozoa) were incubated in amphibian ringer's solution at room temperature. One hundred μ l aliquots of incubation medium were taken at 2 or 4 hr and assayed for testosterone (T) by radioimmunoassay. Two-hour control value of T production per individual testis is 22 ng. Addition of 16 ng, 32 ng, or 64 ng hCG to the incubation medium stimulates T production to levels of 38 to 95 ng. Adding 100 ng of LHRH (without hCG) results in 2-hr T production of 103 ng. When 64 ng hCG and 100 ng LHRH are included in the 2-hr incubation together, an intermediate production level of 80 ng is obtained. Similarly, the values obtained when a dose of 100 ng LHRH is included along with 32 ng or 16 ng hCG are intermediate between the values obtained with LHRH or hCG individually. The 4-hr T production level of *R. pipiens* testis *in vitro* is 20 ng in this study. Adding hCG (16, 32, or 64 ng) raises the production to 60 to 115 ng. The T produced by incubating testis slices with 100 ng LHRH for 4-hr is 124 ng. When both hormones are added, intermediate levels are produced. Parallel results are obtained with the LHRH analogue. A dose of 100 ng stimulates T production at 2 hr to 63 ng and at 4 hr to 83 ng. When the analogue (100 ng) is added along with 16 ng, 32 ng, 64 ng hCG the stimulatory effects of the two hormones are not additive.

These results demonstrate that: first, LHRH and one of its analogues directly stimulate steroidogenesis by the *R. pipiens* testis *in vitro*; secondly, the stimulatory effect of the decapeptide or its analogue is not additive to the stimulatory action of hCG; thirdly, at the time and doses employed, the LHRH analogue is approximately equipotent to LHRH in causing T production by the frog testis *in vitro*.

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A general method, employing arsenazo III in liposomes, for the study of calcium ionophores: Results with A23187 and prostaglandins. CHARLES SERHAN, PAUL ANDERSON, ELIZABETH GOODMAN, ELISABET SAMUELSSON, AND GERALD WEISSMANN.

Multilamellar (MLV) and large unilamellar (LUV) lipid vesicles (liposomes) trap the metallochromic dye arsenazo III (2,7-bis(arsenophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonic acid) in their aqueous compartments. When ionophore A23187 was preincorporated into lipid bilayers of either MLV's or LUV's above 0.001 molar %, addition of Ca to the outside of liposomes produced spectral shifts (max. at 656 nm) characteristic of the $\text{AIII} \cdot \text{Ca}_2$ complex. The method permitted detection of two molecules of A23187 per liposome. Liposomes with A23187 were permselective: Mg or other divalent cations were not translocated. Integrity of liposomes was monitored by addition of excess EGTA which dissociated extra-liposomal $\text{AIII} \cdot \text{Ca}_2$ complexes. Incorporation of A23187 did not enhance permeability of liposomes to glucose, nor did valinomycin or gramicidin provoke Ca uptake. Ca uptake was not influenced by omission of cholesterol from the usual molar lipid composition of MLV's or LUV's (phosphatidyl choline 7: dicetylphosphate 2: cholesterol 1). Since prostaglandins may act as calcium ionophores, we have incorporated into MLV's and LUV's stable prostaglandins (PGE_2 , PGI_2 , PGB_1), endoperoxides (PGH_2 -analogues), and a water-soluble, polymeric derivative of PGB_1 : PGBx . None acted as ionophores. In contrast, when added to the outside of preformed MLV's or LUV's PGBx , at concentrations above one micromolar, provoked permselective Ca uptake equivalent to that induced by 10^{-8} M A23187. These studies demonstrate not only that liposomes containing arsenazo III may be employed in a sensitive assay method to study agents which form channels for divalent cations, but that a water-soluble derivative of a naturally occurring fatty acid, PGBx , is a potent calcium ionophore.

Sulfate-depletion profiles and sulfate-reduction rates for a salt marsh. SUSAN SIEN.

Sulfate reduction is a major process in salt marsh decomposition, and the resulting reduced inorganic sulfur compounds are a potential source of energy to the coastal food web. As a step toward a better understanding of the process of sulfate reduction in salt marshes, the extent of sulfate depletion and the rate of sulfate reduction were measured in several sites in the Great Sippewissett Salt Marsh. Profiles of sulfate depletion in peat over depths up to 23 cm were constructed for various types of marsh sediments: dwarf *Spartina alterniflora*, tall *S. alterniflora*, *S. patens*, and creek bottom. Profiles are highly variable for the sites sampled, with the exception of a creek bottom site where distinctly less sulfate is found at greater depths. Variations were also found among cores taken at the same site. There are two possible explanations for such variability in sulfate depletion profiles: first, the hydrology of these marsh sediments is very complex, affecting the time during which sulfate reduction has occurred, and secondly, the extent of sulfate reduction and re-oxidation varies with depth and vegetation type.

Sulfate reduction rates were measured in the salt marsh at sites containing homogeneous stands of dwarf *Spartina alterniflora*. A method was used to determine the rate of disappearance of sulfate in replicate cores incubated for periods of up to 2 weeks. Rates were consistently high for the top 5 cm of the peat and tended to be much lower at other depths. Although reduction rates varied greatly between similar sites and among replicate cores, they are well within the range of rates of reduction previously determined for dwarf *S. alterniflora* sediments during the summer by a radio-tracer technique. Much of this variation may be due to variation in the initial sulfate concentrations among the replicate cores.

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Repeated genomic sequences cloned from the sea urchin Lytechinus pictus.

JONATHAN SMITH, LESLIE SERUNIAN, WILLIAM PHILLIPS, ANDREW MURRAY, SHARON HOROWITZ, AND GERALD RUBIN.

The sea urchin genome is composed of approximately 25% repeated DNA sequences, most of which are short (average length 300 bases) and well characterized. However, 6 to 8% of the genome consists of repeated elements greater than 2 kilobases in length. Although much attention has been directed toward the tandemly repeated sequences (histone and ribosomal RNA genes), the dispersed repetitive sequences have been virtually ignored, except for studies showing that they are more highly conserved than the short interspersed repeats.

We have studied the long repeated sequences in the genome of *Lytechinus pictus* by isolating and characterizing genomic DNA fragments using recombinant DNA technology. Recombinant plasmids were constructed by ligating Hind III digested *L. pictus* genomic DNA into the Hind III site of bacterial plasmid pBR322 and were used to transform *E. coli*. Eight hundred and fifty transformants were isolated by resistance to ampicillin and screened with a ³²P-labeled probe enriched in long repeated sequences. This probe was prepared by reassociating DNA to low Cot values, digesting with S1 nuclease, size-selecting on sucrose gradients, and nick translating. When the S1-resistant DNA was electrophoresed on agarose gels, discrete bands, 500 and 800 nucleotides in length, were observed. Approximately 100 of the original clones hybridized to the repeated DNA probe; 48 of these were hybridized to ³²P-complementary DNA synthesized from polyadenylated RNA isolated from hatching blastulae and pluteus embryos. One single clone hybridized to both of these probes indicating that the genomic fragment contains both repeated and transcribed sequences. DNA was also prepared from two clones containing long repeats (10 and 13 kilobases in length). The genomic inserts represented by repeated sequences were localized by hybridization of the repetitive DNA probe to restriction fragments of clones transferred to nitrocellulose according to the method of Southern.

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A comparative study of the marginal bands in newt (Notophthalmus viridescens) and chick (Gallus domesticus) erythrocytes. DENICE SMITH.

Although the erythrocytes of newt and chick are like the red cells of other non-mammalian vertebrates in that they possess a microtubule-containing structure called a marginal band, these cells differ considerably in cell size and numbers of microtubules present in the marginal bands and they additionally differ in the degree to which the marginal band can be isolated from the nucleus, suggesting that the marginal bands in the two systems might be organized quite differently.

While marginal bands from chick erythrocytes are bound tenaciously to the nucleus by a fibrous matrix, newt marginal bands can be released from the nucleus (and possibly the matrix) by treatment of the erythrocytes with 0.5% Triton X-100 followed by a separation procedure in a sucrose step gradient. Analysis of these preparations by SDS PAGE reveal proteins which migrate in regions corresponding to MAPs, spectrin, tubulin, and actin when compared to standards known to contain these proteins, suggesting a possible association of marginal bands with the cytoskeleton.

Treatment of marginal bands with 0.5 M KCl, which is known to disrupt the trans-band material, permits the individual microtubules of chick marginal bands to be visualized in negative stained preparations. When newt marginal bands are treated with 0.5 M KCl for 1 hr, the marginal bands appear under phase contrast microscopy to be released from the cell, with microtubules splaying apart. Preliminary analysis by SDS PAGE suggests a selective removal of high molecular weight proteins by this procedure. Treatment of chick and newt erythrocytes with DNase (1mg/ml) for 1 hr appears to release the marginal band from the nucleus. Although the fibrous matrix appears to be disrupted, the marginal bands retain their characteristic curvature. Preliminary analysis of the preparations by SDS PAGE suggests a selective removal of high molecular weight proteins as well as spectrin.

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Heavy metal effects on intestinal absorption of nutrients in the toadfish, Opsanus tau. ROBIN SOCCI, JOHN MERCURO, AND A. FARMANFARMAIAN.

Heavy metals (Cd, Hg, Cr, Co) have been shown to inhibit the activity of various enzymes in different animal and plant tissues. Less is known of the inhibitory effect of these heavy metals on membrane transport mechanisms and, in particular, intestinal epithelium which acts as the first and most important barrier to the entry of these heavy metals in higher invertebrates and all vertebrates. We have examined the effect of CdCl_2 on the absorption of amino acids in the intestine of the toadfish *in vivo*.

The test solution consisted of Forster Taggart fish ringer, a ^{14}C -labeled amino acid (L-alanine, L-leucine), with or without a chosen level of the heavy metal inhibitor (CdCl_2). A closed loop of the midgut was cannulated under anesthesia and the test solution inserted for a 10 min incubation period. This solution was mechanically mixed by an oscillating pump stirrer. Two concentrations of substrate were usually used. Tritiated inulin was included as a water marker and radioisotopes were measured by liquid scintillation spectrometry. At 40 mM L-leucine and $5 \times 10^{-4}\text{M}$ CdCl_2 , there was a reduction in the absorption rate of 36% ($P \leq 0.01$), compared to that of the control without CdCl_2 . However, at the 6.6 mM level, no significant inhibition was observed. When the CdCl_2 level was reduced to $1 \times 10^{-4}\text{M}$, uptake of L-leucine was reduced by 52% ($P \leq 0.001$). At $1 \times 10^{-5}\text{M}$ CdCl_2 , there was no significant ($P \leq 0.05$) inhibition of leucine transport. Two concentrations of CdCl_2 (1×10^{-4} and $5 \times 10^{-4}\text{M}$) were tested with 6.6 mM L-alanine. In both cases there was no significant ($P \leq 0.05$) cadmium inhibition.

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Behavioral evidence for two populations of amino acid receptors in catfish taste. ANN STEWART, BRUCE BRYANT, AND JELLE ATEMA.

From electrophysiological studies on the maxillary barbel of the catfish, it appears that there are two populations of receptors for amino acids: a generalist and a specialist (Caprio, J. and D. Tucker, 1976, *Soc. Neurosci.*, 2: 152). The generalists respond best to alanine, but also to all other amino acids, while the specialists respond only to L-arginine, its methyl-ester, and slightly to alanine. In cross-adaptation experiments, a 10^{-4}M solution of alanine raised the threshold of all amino acid responses except arginine to about 10^{-3}M ; conversely an adapting arginine background had a minimal effect on the thresholds for other amino acids.

We tested behaviorally by touching the maxillary barbels of eight resting catfish (*Ictalurus nebulosus*) with cotton swabs dipped in stimulus solutions. The fish were first conditioned to not respond to the tactile stimulus of a blank swab. Then thresholds for alanine and arginine were determined. Both ranged from 10^{-6} to 10^{-8}M . When the aquaria were flooded with a 10^{-4}M background of arginine, the alanine threshold was raised by 0.7 or more log units in four fish and unchanged in four. When 10^{-4}M alanine was used as the adapting background, increases of 0.7 log units or greater were found for six fish ($P < 0.05$, sign test). Thus the alanine effect on arginine thresholds was slightly larger than the converse effect. When arginine and alanine stimuli were presented in their own background, the stimuli had to be 5 to 25% above background to elicit responses. This behavioral study confirms earlier electrophysiological work and demonstrates that catfish have an alanine and an arginine taste system which operate rather independently. Such a system would make it possible to detect arginine in high backgrounds of other amino acids. The significance for the catfish's natural environment has yet to be determined.

Evidence for three conducting systems in the hydroid Clava. DARRELL R. STOKES AND NORMAN B. RUSHFORTH.

Three nonpolarized conducting systems can be activated by electrical stimulation of the colonial hydroid *Clava squamata*. Two of these systems have been described within individual polyps: a Contraction Pulse System (CPS) and a Delayed Burst System (DBS). Stimulation of the CPS produces Contraction Pulses (CPs) which may occur as single events or as

bursts of potentials of facilitated amplitude (about 0.5 mV), and are correlated with polyp contraction. Stimulation of the DBS produces a program of bursts of pulses (DBPs) beginning about 20 sec after a shock. DBPs facilitate within a burst to amplitudes of about 0.5 mV and correlate with symmetrical depression of the tentacles.

Electrical stimulation of the stolons which interconnect polyps of the colony activates a third system: a Fast Pulse System (FPS). Fast Pulses (FPs), with no behavioral correlate, are of smaller amplitude (ca. 0.05 mV) and shorter duration (ca. 20 msec) than pulses from the CPS or DBS. Such pulses are conducted at a velocity of about 10 cm/sec, similar to that of the DBPs but three times faster than CPs. Production of FPs by electrical stimulation of the stolons induces CPs in adjacent polyps. In contrast, stimulation of a polyp to produce CPs does not evoke FPs in the stolons. This suggests that polarized transmission junctions occur between the stolons and the polyps.

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Statocyst cilia transmit rather than transduce mechanical stimuli. E. W. STOMMEL,
R. E. STEPHENS, AND D. L. ALKON.

We have investigated the role of motile cilia (hairs) in mechanotransduction by the statocyst of the nudibranch mollusc *Hermisenda crassicornis*. Movement of hairs which experience the weight of statoconia causes variance of voltage noise and membrane depolarization. Two complementary approaches were used to immobilize the cilia. Vanadate anion (V^{5+}) was iontophoretically injected into hair cells, using 0.1 nA currents through an electrode filled with 0.01 M $NaVO_3$ and 0.1 M Na-acetate. This reversible inhibitor of dynein cross-bridge cycling initially caused the cilia to lose their rigid, vibratile motility and assume a more classic, pliable beat pattern. When the concentration of vanadate approached 10^{-6} M, the cilia were arrested against the cell membrane. Voltage noise decreased as the cilia slowed and bent more extremely, nearly disappearing as motility was lost. The cell no longer depolarized upon gravitational or mechanical stimulation. Rapid reversal with norepinephrine or slow reversal with time restored both the noise and depolarization response. Cilia were also rendered rigid by covalent cross-linkage of the membrane "sleeve" to the 9 + 2 axoneme, using the photoactivated, lipophilic, bifunctional agent 4,4'-dithiobisphenyl azide. In initial stages of cross-linkage, the cilia remained vibratile but slowed and moved through wider excursions. Voltage noise decreased in frequency but increased in amplitude. When the cilia were fully arrested, voltage noise was minimized while the resting potential remained essentially constant. Since cilia which are partially arrested by vanadate undergo considerable bending but show decreased noise, neither the axoneme nor the ciliary membrane proper appear to be sites of transduction. In full vanadate arrest, the exposed plasma membrane itself shows no response to stimulation. However, in beating, stiffened cilia, voltage noise becomes amplified, implying an increased efficiency of transduction. Therefore, the basal region is the most likely transduction site, being the leverage point to which force is applied via the ciliary shaft.

The larval stages of Lepocreadium areolatum (Linton, 1900) Stunkard, 1969,
(Trematoda: Digena). HORACE W. STUNKARD.

At the General Scientific Meetings a year ago, it was reported that certain hydrozoan and scyphozoan medusae and ctenophores in the Woods Hole area harbor uncysted metacercarial stages of digenetic trematodes. Five species have been recognized and the life-cycles of three of them have been worked out and published: that of *Neopoechona pyriforme* Linton, 1900) Stunkard, 1969 by Stunkard (1969, *Biol. Bull.*, **136**: 96-113), of *Lepocreadium setiferoides* (Miller and Northup, 1926) Martin, 1938 by Stunkard (1972, *Biol. Bull.*, **142**: 326-334); and of *Lintonium vibex* (Linton, 1900) Stunkard and Nigrelli, 1930 by Stunkard (1978, *Biol. Bull.*, **155**: 383-394). The discovery on 20 July 1978 of an ophthalmotrichocercous cercaria from *Nassarius trivittatus*, whose structure agreed with an unidentified metacercaria, first noted in 1973, led to the elucidation of the life-cycle. The incidence of infection by this species is very light; the cercaria had not been found before in the examination of thousands of

N. trivittatus. Only one infected snail was found in 1978 and only one in the examination of hundreds of snails in 1979. The adult worm produces very few, very large eggs. A happy ecological situation facilitated experimental procedure. Crowell (1945) reported that the hydroid, *Podocoryne carnea*, lives on the shells of *N. trivittatus*. This snail harbors the trematode infection and liberates cercariae; the hydroid liberates medusae, which are attacked and invaded by the cercariae. Medusae, infected in the laboratory and embedded in bits of squid and clam, were fed to cunners, *Tautoglabrus adspersus*. This method yielded juvenile and gravid specimens of *Leporeocodium arcuatum*, reported initially from white perch and cunners by Linton (1900, *Bull. U. S. Fish Commission* (1898) : 267-304; 1901, *Bull. U. S. Fish Commission* (1899) : 405-492) and known also from puffers and other fishes since that time.

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Pancreatic secretion granules are associated with microtubules. K. A. SUPRENANT
AND W. L. DENTLER.

Secretion granules (SG) isolated from goosefish pancreatic islets specifically associate with microtubules assembled *in vitro* from chick brains. Microtubules and SG's were mixed and associations were monitored in solution by darkfield light microscopy. Associations were confirmed by electron microscopy. SG's only bound to microtubules with high molecular weight associated proteins (MAP-MT's). In the presence of 0.2 mM ATP, SG's bound to 43% of the MAP-MT's. Upon addition of 100- μ M cAMP, 57% of the MAP-MT's bound SG's. Microtubules also aggregated in the presence of cAMP to form large bundles associated with granules. The addition of 5-mM ATP to the cAMP treated aggregates, released the SG's from the MAP-MT's and dissociated the MT aggregates. Only 12% of these MAP-MT's had granules bound. The release of the SG's and the dispersion of the MT's are nucleotide specific, since pyrophosphate, AMP, ADP, GTP, and ITP have no effect on the release or dissociation. Polystyrene beads did not associate with the MT's under any assay conditions. The association between SG's and MAP-MT's was strong enough to be maintained during ultracentrifugation. SG's and MAP-MT's were mixed in 2M sucrose and centrifuged through a step gradient. The granules moved upward in the gradient with MAP-MT's associated to the SG membrane. In thin section, the association appeared to be mediated by the filamentous MAP proteins. Intact MT's from goosefish islets were isolated in the presence of hexylene glycol. SG's were attached along the length of the microtubules as assayed by negative staining.

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The living state. ALBERT SZENT-GYÖRGYI.

"What is life" is the main problem of biology. We can ask the same question in a more meaningful way: what is the difference between animate and inanimate. The difference is so great that we are justified to speak of the "living state" as a special physical state. Material systems can be decomposed into molecules, molecules into atoms, atoms into electrons and nuclei. Electrons and nuclei can be decomposed still further but the energies needed are far beyond biological dimensions. The great sensitivity and reactivity of living systems indicate that the smallest independently moving particles are very small. We have to look for them among electrons and nuclei.

The number of electrons of the electron cloud, is given by the atomic numbers. With this number of electrons the electronic cloud is closed, the atom is a "closed shell" atom. Closed shell atoms build closed shell molecules. In them the electrons have to be immobile. To make them mobile electrons have to be taken out. Electrons cannot be taken out without disturbing the electroneutrality. Electrons can be transferred to another atom by charge transfer, and form then a charge transfer complex which is electroneutral. In the one of the two atoms or molecules the cloud will be desaturated and electrons will have a mobility. Charge transfer has been studied on various models having methylglyoxal as electron acceptor and methylamine, or symmetrical dimethylethylenediamine. The properties of these models are

discussed. Charge transfer introduces an electronic mobility and reactivity, as shown by the optical and electron spin resonance spectra. The living state is an electronically desaturated state.

Migration rate of mud bacteria as a function of magnetic field strength. BARBARA D. TEAGUE, MICHAEL GILSON, AND AD. J. KALMIJN.

Certain marine and freshwater mud bacteria are endowed with a permanent magnetic dipole moment. This moment is attributed to an endogenous chain of tightly coupled, single-domain magnetite crystals. When separated from the mud, these magnetic bacteria swim north, following the earth's magnetic field lines. As at Woods Hole, Massachusetts, the field lines are steeply vertically inclined, the bacteria rapidly return to the bottom substrate where they seem to thrive best. To quantify this migration, we measure the time to traverse the distance between two lines, 1 mm apart, as a function of the ambient magnetic field strength. Using dark-field illumination, we observe single organisms as they migrate in a low-oxygen hemocytometer chamber. We control the ambient magnetic field by regulating the current through a Helmholtz-coil system. At high magnetic field strengths, the bacteria follow a virtually straight path, swimming at rates around 150 $\mu\text{m/sec}$. At lower field strengths, they take a more random path which reduces their migration rate. Although they swerve moderately at the earth's magnetic field strength (0.5 gauss), the bacteria still achieve about 80% of their maximum migration rate observed at higher-gauss fields. This suggests that the bacterial dipole moments are well adapted to orientation in the earth's magnetic field. Since the strength of their magnet determines the degree to which the organisms overcome random motion, we can estimate the magnitude of their dipole moment.

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Excitation of squid giant axon membrane exposed to an identical solution intracellularly and extracellularly. SUSUMU TERAKAWA.

An electrical excitability was demonstrated in a squid giant axon membrane exposed to an identical solution intracellularly and extracellularly. The solution used for the intracellular and extracellular perfusion contained glycerol (12% in volume) and one of cobalt-, manganese-, barium-, or nickel-salts at the concentration of 2 mM. Usually, nothing else was added to the solution. The membrane potential measured by a KCl-containing glass pipette electrode stayed in the range of -10 to +10 mV during the intracellular and extracellular perfusions with an identical solution. When a constant inward current was passed through the membrane under such perfusions, oscillatory changes in the membrane potential and the membrane conductance were observed. The shape and the time course of these changes were very similar to those of repetitively fired action potentials. When calcium-, magnesium-, or strontium-salt was used as a sole electrolyte species, the oscillatory response did not appear. The oscillatory response was reversibly suppressed by 4-aminopyridine, but could not be suppressed either by tetrodotoxin or by D-600. A voltage clamp study revealed the N-shaped I-V characteristic of the membrane. Interpretations proposed by Teorell (1959, *J. Gen. Physiol.*, 42: 847) and by Katchalsky (1968, *Q. Rev. Biophys.*, 1: 127) for oscillatory phenomena in inanimate membranes may be helpful in understanding the results obtained.

Comparison of long-lasting hyperpolarization produced synaptically with that induced by cyclic AMP in Aplysia pacemaker neurons. STEVEN N. TREISTMAN.

Cell R15 of *Aplysia* possesses an endogenous rhythm in which bursts of action potentials alternate with interburst hyperpolarizations. A few stimuli to the branchial nerve can silence the cell for periods of minutes to hours. We had previously shown that phosphodiesterase inhibitors could augment the hyperpolarization evoked by branchial nerve

stimulation, suggesting that cyclic nucleotides may play a role in the generation of these long-lasting potentials. Intracellular injection of cAMP derivatives, as well as GMP-PNP, an adenylate cyclase activator, caused sustained hyperpolarization of R15, strengthening this hypothesis. In this presentation, a voltage clamp paradigm is used to generate the pseudo-steadystate current-voltage curve, and compare the conductance changes produced by nerve stimulation with those induced by cAMP manipulation. Branchial nerve stimulation at high stimulus strengths caused an increased slope conductance in the R15 plot. Moreover, the negative slope region in the plot thought to be basic to generation of bursting activity was obliterated. Similar results were obtained after intracellular injection of GMP-PNP, cAMP derivatives, or cAMP in cells pretreated with the phosphodiesterase inhibitor, Ro 20-1724. These results are compatible with a cAMP mediation of the long-lasting synaptic event seen in R15 after branchial nerve stimulation.

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Mechanism of red algal bile pigment formation. ROBERT F. TROXLER, STANLEY B. BROWN, AND KEVIN M. SMITH.

The photosynthetic accessory pigment phycocyanobilin is structurally closely related to bilirubin, the end product of heme turnover in mammals. The latter process involves insertion of two oxygen atoms and ^{18}O -labeling experiments have shown that these atoms are derived from two oxygen molecules (Two-Molecule Mechanism) rather than from one oxygen molecule (One-Molecule Mechanism). The similarity in structure between bilirubin and algal bile pigments suggests that heme may be an intermediate in phycocyanobilin biosynthesis. However, since plants manufacture substantial quantities of magnesium protoporphyrin IX, it has been suggested that phycocyanobilin might arise from the magnesium branch of the porphyrin pathway.

We have performed preliminary ^{18}O -labeling experiments with the unicellular rhodophyte, *Cyanidium caldarium*, which suggested phycocyanobilin synthesis by the Two-Molecule Mechanism. However, photosynthetic production of $^{16,18}\text{O}_2$ prevented a precise quantitative interpretation of the results. In the present work, ^{18}O -labeling experiments were performed using cells in which photosynthesis was inhibited with the inhibitor, 3-(3,4-dichlorophenyl)-1,1-(dimethylurea). Quantitative measurements on the mass spectra of phycocyanobilin isolated from these cells, revealed high ^{18}O incorporation and clearly demonstrated synthesis by the Two-Molecule Mechanism. This process probably involves independent attack by two oxygen molecules followed by intramolecular rearrangement and release of an oxygen molecule containing atoms from each of the attacking oxygen molecules.

To evaluate the possible origin of phycocyanobilin via the magnesium branch of the porphyrin pathway, a chemical model system was studied in which a magnesium chlorin was converted to a dihydrobiliverdin derivative, chemically analogous to phycocyanobilin. A thin-layer chromatography system was developed by which the product could be purified and isolated for mass spectrometry. ^{18}O -labeling experiments on this model system are in progress. If the reaction occurs by the One-Molecule Mechanism, then phycocyanobilin synthesis via the magnesium branch is very unlikely. If, however, a Two-Molecule Mechanism is found, further experiments to distinguish between the magnesium and iron branches of the porphyrin pathway will be necessary.

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Glial-axonal protein transfer: its functional significance. MICHAEL TYTELL AND RAYMOND J. LASEK.

In the squid giant axon, many proteins synthesized by the adaxonal glial sheath cells are actively transferred into the axon. We examined these transferred proteins and compared them with the proteins of axoplasm (AXM) and the stellate ganglion of the giant axon. Proteins synthesized by the sheath and ganglion were labeled by incubating the giant

axon and ganglion in 1 mCi/ml ^3H -leucine in artificial sea water for 60 to 360 min at 19 to 21° C. Then the AXM, containing labeled transferred proteins, was extruded. The empty sheath, AXM, and ganglion were each homogenized in either a physiological buffer or electrophoresis solubilization buffer. The homogenates were analyzed by ultracentrifugation, Sephadex column chromatography, and 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

The centrifugation and column chromatography experiments revealed that the proteins transferred into the AXM were contained in a particle which was unaffected by hypotonic shock or 400 mM KI, but was partially disrupted by 1% Triton and completely disrupted by 0.1% SDS. The 2D-PAGE analysis of the transfer particle in AXM showed that it consisted of a select group of about 50 sheath proteins, one of which has been tentatively identified as actin.

Eleven of the transfer particle proteins in the 2D-PAGE analysis were coincident with and therefore, identical to major axonal proteins. Since axonal proteins are made in the soma and supplied to the axon via axonal transport, we compared the 2D-PAGE pattern of proteins synthesized in the ganglion with these 11 transfer particle proteins. All but one were also made in the ganglion. Thus, some of the major proteins of axoplasm, presumably supplied by the soma, are also selectively transferred to the axon in particulate form from the adaxonal glial cells. We propose that glial-axonal protein transfer may serve to supplement a specific fraction of axonally transported proteins.

RNA synthesis in isolated chloroplasts. ELIZABETH VIERLING.

Chloroplasts were isolated from *Hordeum vulgare* cv. Himalaya (barley) using 10 to 80% preformed Percoll silica sol gradients. These gradients separate intact plastids from broken plastids, mitochondria and other cellular debris. As judged by phase contrast microscopy the preparations were free of nuclear and bacterial contamination. Chloroplasts were prepared for electron microscopy using the Miller spreading technique after a brief lysis in 0.005% Triton X-100. DNA fibrils observed at the periphery of lysed chloroplasts were morphologically distinct from both eukaryotic chromatin and prokaryotic DNA. DNA strands had a diameter several times that of double-stranded DNA and were irregularly beaded. Structures which could be identified as transcriptionally active were not observed. For *in vitro* transcription, chloroplasts were incubated in the presence of ^3H -UTP at a chlorophyll concentration of 100 $\mu\text{g}/\text{ml}$. Incorporation of ^3H -UTP into an RNAase-A-sensitive product proceeded linearly for 30–45 min. Incorporation was α -amanitin insensitive at a concentration of 10 $\mu\text{g}/\text{ml}$ and was not light dependent. The time course and level of RNA synthesis were identical with or without the addition of 1 mM MnCl_2 . This observation is consistent with chloroplast but not with nuclear RNA polymerase activity. Initiation of transcription was assayed for using Hg-agarose affinity chromatography of γ -thiol ATP or GTP labelled RNA. Results indicated that 0.4% of the ^3H -UTP incorporated represented new transcript initiation.

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Plasma fibronectin (CIG) of the dogfish plasma mediates attachment of phagocytes to collagen substrates. GERALD WEISSMANN, JAMES W. LASH, G. E. SIEFRING, J. IBERS, AND LASZLO LORAND.

Plasma fibronectin (cold-insoluble globulin CIG) mediates the attachment of mammalian cells to collagenous substrates: we sought evidence for a similar protein in plasma of *Mustelus canis*. Blood phagocytes of the dogfish (10^6) were exposed either to uncoated Sepharose 4B beads (10^4) or to beads coated with gelatin by means of cyanogen bromide. In heparinized, complement-sufficient plasma, 58 and 90% of control and gelatin beads, respectively bound three or more phagocytes (30 min, 23° C); evidence that complement activation (as in human plasma) by Sepharose 4B mediates cell adhesion to polysaccharide particles. In de complemented (30 min, 56° C) plasma, only 4% of control, but 75% of gelatin beads had phagocytes attached. When plasma was also depleted of CIG (by preincubation with gelatin beads), cells attached to 5% of control, and to only 28% of gelatin beads. Small petri dishes were prepared with

purified mammalian collagens (Types I, II, and III) with and without proteoglycan aggregates. Dogfish phagocytes in de complemented plasma avidly adhered to each type of collagen (but not to uncoated areas of the dish) and proteoglycans diminished attachment. Dogfish CIG was isolated by affinity chromatography on Sepharose 4B-gelatin and eluted in 2M arginine; yield: 0.38 mg/ml plasma. After dialysis, the isolated protein migrated identically to human CIG on 3.8% polyacrylamide gel electrophoresis (MW = 220,000) and dissociated to a single chain in the presence of dithiothreitol. Gelatin—but not control—beads exposed to either de complemented dogfish plasma or purified CIG showed specific fluorescence when first reacted with goat anti-human CIG or rabbit anti-hamster fibroblast fibronectin and then with rabbit anti-goat IgG. Data suggest that CIG is conservative both in structure and function: attachment of dogfish and mammalian phagocytes is mediated by the same recognition protein.

Protein synthesis in cell free extracts of Lytechinus pictus eggs. MATT WINKLER, ELLEN BAKER, AND TIM HUNT.

The rate of protein synthesis increases 20- to 30-fold upon fertilization of the sea urchin egg as maternal mRNA is mobilized into polysomes. The increase in intracellular pH accompanying fertilization appears to play a major regulatory role in this activation. A cell-free protein synthesizing system prepared from *Lytechinus pictus* eggs was used to study this regulatory process. These cell-free systems show excellent incorporation of amino acids into protein after an initial, somewhat variable lag. Raising the pH shortens this lag and appears to mimic the activation of protein synthesis *in vivo*.

These systems will translate added globin, histone and TMV mRNAs at both pH's, as measured by the appearance of new labeled bands on SDS gels. The added mRNAs, like the endogenous mRNAs, are translated to a much lesser extent at pH 6.9 than at 7.4. Addition of even large amounts (up to 320 $\mu\text{g/ml}$) of histone mRNA results in at most a 2-fold stimulation of protein synthesis at pH 6.9 and some degree of inhibition at pH 7.4. Analysis of products suggests that the added mRNA competes with the endogenous mRNA at pH 7.4, while it augments existing protein synthesis at pH 6.9. Previous analysis of the protein synthetic machinery in the intact egg has been interpreted to indicate that the maternal mRNA is unavailable for translation before fertilization; but the very modest stimulation of protein synthesis by added mRNA at pH 6.9 seems inconsistent with this idea, and suggests that other components of the translational machinery may be in a dormant state before fertilization. The transit time for a specific mRNA was measured in this system by the addition of TMV mRNA and assaying for the first appearance of completed product on SDS gels. The maximum transit time for the synthesis of the 130,000 MW product is 20 min at pH 7.4, or approximately one amino acid per second.

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Low level excitation of chlorotetracycline fluorescence in Haemaphysalis endosperm cells using image intensification. STEPHEN M. WOLNIAK, PETER K. HEPLER, WILLIAM T. JACKSON, AND GEO. T. REYNOLDS.

Cell plate formation in dividing endosperm cells of *Haemaphysalis* involves the migration of calcium-rich vesicles to the equatorial region of the cell, followed by their coalescence at the site of incipient wall deposition. The calcium-membrane probe, chlorotetracycline (CTC, 10-50 μM) permits visualization of the plate-forming vesicles in living cells with fluorescence microscopy. CTC fluorescence is punctate and is localized at the spindle poles from prophase through late anaphase. Previous studies have shown that the poles are enriched in endoplasmic reticulum, mitochondria and plastids. By the onset of telophase, there is a dispersion of fluorescence at the poles and a concomitant increase in the region of the forming plate. Since visualization of fluorescence with intense near-UV light (390-410 nm) often disrupts normal divisions, we reduced incident excitation light by a factor of approximately 250 with a neutral density filter. We then employed image intensification of the fluorescence emission to study plate deposition as a dynamic process in living cells. Fluorescence micrographs were

taken at 15 min intervals during anaphase and telophase. In agreement with the high intensity fluorescence images, we first observed an increase in spindle midzone fluorescence when the poleward migration of the kinetochores was only 70 to 90% complete. At this stage of division, trailing chromatids extend through the equatorial region of the spindle. CTC-fluorescent material appears to aggregate at numerous sites in the phragmoplast, and as chromatid migration progresses, the highly fluorescent plate thickens. This technique demonstrates the feasibility of image intensification fluorescence studies on living cells that are normally adversely affected by intense short wavelength light (Reynolds, 1979, *Biol. Bull.*, **157**: 391).

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Characterization of the hemoglobin of the clam, Astarte castanea. THOMAS D. YAGER AND K. E. VAN HOLDE.

The hemoglobin of the clam *Astarte castanea* is a large (10^7 dalton) protein which binds oxygen non-cooperatively. At pH ≥ 9 in the absence of divalent cations, this protein dissociates into a single subunit.

Clams were placed on ice 10 min and then easily opened by severing the adductor muscles. At 4° C, hemolymph was collected from the pallial cavities by pipette, centrifuged 7 min at $13,000 \times g$, scrubbed with CO and dialyzed against tris or bicarbonate containing 10 mM EDTA. The dialysate is a virtually pure solution of the subunit. Polyacrylamide gel-electrophoresis in SDS revealed one band at 340,000 daltons, with a trace higher-MW contaminant (the sample having been boiled in 1% SDS/ β ME for 2 min and run upon 7.7×2.6 and 3.1×2.6 Laemmli slabs). Analysis in the ultracentrifuge gave $S_{20,w}^\circ = 11.3$ S and, for the concentration-dependence of S , $k_s = 45$ cm²/g (with $\bar{v}$ assumed to equal 0.7425 cm³/g).

Purification of the subunit was achieved by molecular sieve chromatography of the dialysate (through Bio-Rad A-1.5 m beads, mesh 200, on an 80×1.6 cm Pharmacia column). A single eluent peak was observed, for which the ratio of 414/280 nm absorbance was constant at 2.3. The trailing edge of the peak was homogeneous in sedimentation velocity and in SDS/PAGE (with the sample heated to 60° in 1% SDS/ β ME for 2 min, and run upon a 5.2×2.6 Laemmli slab). Sedimentation equilibrium analysis also showed a single component, with MW = 342,000. Using the $S_{20,w}^\circ$ and MW values from ultracentrifugation, the frictional coefficient was calculated to be 1.50. This relatively high value indicates that the subunit has some asymmetry.

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In vitro reassembly of squid brain neurofilaments and their purification by assembly-disassembly. ROBERT V. ZACKROFF, ANNE E. GOLDMAN, AND ROBERT D. GOLDMAN.

Neurofilaments from squid brain tissue were disassembled at high ionic strength and reassembled upon lowering the ionic strength to physiological levels. Brains were homogenized in and extracted with disassembly buffer (1 M KCl, 0.25 M MES, 5 mM EGTA, 1 mM PMSF, pH 6.6) and centrifuged at $250,000 \times g$ for 90 min at 4° C. The resulting supernatant contained soluble subunits which rapidly reassembled into neurofilaments upon dilution with 9 volumes of reassembly buffer (0.25 M MES, 5 mM EGTA, 1 mM PMSF, pH 6.6). Reassembled neurofilaments harvested by centrifugation consisted of one major polypeptide of approximately 60,000 daltons, and three minor polypeptides of higher molecular weight which were barely detectable on normally loaded (15 μ g) slab gels. Further purification by a second cycle of disassembly, clarification, and reassembly resulted in retention of all four polypeptides. Negative staining of 1-cycle purified and subsequently depolymerized neurofilaments revealed the presence of ca. 3 nm diameter, 5 to 25 nm long subunits. Upon dilution into reassembly buffer, the turbidity increased abruptly and 10-nm diameter filaments appeared. This abrupt turbidity increase was followed by a slower turbidity rise to plateau which was

correlated with neurofilament elongation. Reassembled neurofilaments exhibited the light-scattering properties of long rods, indicating that turbidity may be used as a measure of polymer weight concentration. In addition to neurofilaments, negative staining revealed the presence of dense structures from which filaments often appeared to radiate. The dense structures were also present after salt-induced disassembly of neurofilaments, and were retained through at least two assembly-disassembly cycles. These results constitute the first successful purification of neurofilaments by assembly-disassembly. Analysis of possible homologies of neurofilament polypeptides and polypeptides of intermediate filaments from non-neural cells is in progress.

This work was supported by NSF and the Runyon-Winchell Cancer Fund.

Bacterial denitrification: a gas chromatographic study using acetylene inhibition of N_2O reductase in two hour incubations. C. M. ZACKS, P. A. STEUDLER, AND J. M. MELILLO.

Current techniques for measuring denitrification potential in soil by the acetylene block method require incubation times of many hours or days. We have determined that denitrification characteristics of soil change rapidly after sampling, suggesting that misleading rates will be obtained unless the incubation is initiated within a day of collection, and is completed as soon as possible. A system was developed to measure denitrification in unaltered field samples in a 2-hr period. This was made possible by detection of N_2O in the 0.02–0.2 ppm range by electron capture, and use of N_2 instead of He as the balance gas in incubation vessels. In this range, short-term incubations required greater reproducibility of N_2O concentration values than could be obtained with a N_2O -He mixture. Improvement was noted when N_2 was used in place of He, which was attributed to improved homogeneity of the N_2O - N_2 system over N_2O -He.

In preliminary studies of a forest ecosystem, soil from clearcut and undisturbed areas was assayed without further laboratory alteration. It was found that activity in clearcut areas was up to 30 times greater than in the undisturbed control (650 pg N_2O -N/hr vs. 20 pg N_2O -N/(hr·g organic wt)). This large difference was confined to surface layers alone. Correlations were found between denitrification potential and the concentration of mineral nitrogen ions, as determined by extraction with 2N KCl. Nitrate-nitrite maintained a constant low level over a range of denitrification rates, suggesting rapid nitrate uptake, while ammonium concentrations were found to be high in high potential areas, and low in inactive sites. Rapid uptake of nitrate is supported by the finding that addition of this ion induced dramatic increase in activity, but further investigation is required to assess the relative contributions of nitrate and ammonium precursors to the denitrification pathway.

Fetatures of elasmobranch eye lenses relative to those of humans. SEYMOUR ZIGMAN AND TERESA PAXHIA.

Comparisons of elasmobranch lenses with those of humans reveal differences in size, visual accommodation and pigmentation and similarities in growth processes and structural protein distribution. Most similar is the age-related buildup of highly aggregated proteins that derive from lower molecular weight precursors. These aggregates are firmly bound to the fiber cell membranes, which are structurally similar in both species. Dogfish (*Mustelus canis*) lenses remain transparent in spite of the presence of these aggregated complexes which would cause light scattering in human lenses. Visibly fluorescent pigment is present at very low levels in dogfish lenses, at intermediate levels in those of surface-swimming sharks, and at very high levels in human lenses, especially in the membrane complexes. Lens fiber cells of elasmobranchs have similar shapes and knoblike interdigitations to those of man, but are several times larger. Their membranes accumulate soluble crystallins which cannot be removed by extensive washing with 8M urea. The major SDS-solubilized membrane protein

of dogfish lenses has a molecular weight of 25,000 daltons, but this may include a certain amount of soluble crystallins. Heating in SDS does not aggregate this protein fully. Near-UV light exposure of lens homogenates in the presence of 1 mM tryptophan increased the binding of protein and non-tryptophan fluorescence to dogfish fiber cell membranes substantially, and electron microscopic examination revealed much greater electron density. The data suggests that additional binding of non-membrane protein to lens membranes could result from exposure of lenses to near-UV light, with fluorescent and pigmented tryptophan photo-products serving as crosslinking agents.

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of biological journal titles is that published each year by BIOLOGICAL ABSTRACTS (BIOSIS List of Serials; most recent issue, 1976). Foreign authors, and others who are accustomed to use THE WORLD LIST OF SCIENTIFIC PERIODICALS, may find a booklet published by the Biological Council of the U.K. (obtainable from the Institute of Biology, 41 Queen's Gate, London, S.W.7, England, U.K. at £0.65 or \$1.75) useful, since it sets out the WORLD LIST abbreviations for most biological journals with notes of the USASI abbreviations where these differ. CHEMICAL ABSTRACTS publishes quarterly supplements of additional abbreviations. The following points of reference style for THE BIOLOGICAL BULLETIN differ from USASI (or modified WORLD LIST) usage:

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Continued on Cover Three

THE BIOLOGICAL BULLETIN

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A FIELD STUDY OF GROWTH AND REPRODUCTION IN *APLYSIA CALIFORNICA*¹

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Some aspects of reproduction in the marine opisthobranch *Aplysia californica* have been the subject of considerable research over the past ten years. Description of a cluster of neurosecretory cells in the central nervous system was followed by extensive work directed toward characterization of their secretory product, which was found to induce egg-laying (Arch, 1972; Arch, Earley, and Smock, 1976; Kupfermann, 1967, 1970; Pinsker and Dudek, 1977). Strumwasser, Jacklet, and Alvarez (1969) noted a yearly cycle both in egg-laying hormone production and in receptivity to the substance. Thus initial investigation of the reproductive cycle in *A. californica* was from the standpoint of its neurohormonal control.

To date, no long-term studies of natural populations of this species have been published. *Aplysia punctata*, found in the eastern Atlantic, has been more extensively investigated. A 16-month study of this species from Trearddur Bay, Anglesey (Carefoot, 1967a) led to the conclusions that *A. punctata* is an annual, breeding between May and October, with major settling in the autumn. The red alga *Plocamium coccineum* supplies a substantial part of the diet of the Trearddur Bay population (Carefoot, 1967a), in addition to supporting more rapid growth than several other algal species being consumed (Carefoot, 1967b, c).

A major difference between *A. punctata* and *A. californica* is size. While *A. punctata* rarely exceeds 80 g (Carefoot, 1967a), *A. californica* often exceeds 3000 g with a species record of 6800 g reported by MacGinitie and MacGinitie (1968). The growth rate and life span necessary to achieve such size have not been documented, nor have seasonal changes in abundance, weight, or reproductive

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activities of a natural population been reported. This paper describes the results of such an investigation.

MATERIALS AND METHODS

This study was conducted between July 1972 and August 1975 at the University of California's Catalina Marine Science Center on Santa Catalina Island, 25 km off the California coast south of Los Angeles. Two field study areas were selected which were in close proximity to the marine laboratory and known to support subtidal populations of *A. californica* (subsequently referred to as *Aplysia*). Site 1 covered part of Bird Rock, a small island approximately 500 m from shore. The area investigated covered about 35 m of shoreline and extended to a depth of 8 m. The total area of ocean floor studied was roughly 5600 m². The sea bed sloped gently within the study area, but dropped more precipitously around the rest of the rock island.

Site 2 consisted of approximately 140 m of coastline starting inside Big Fisherman's Cove on Catalina Island, extending a short distance east along the coast and to a depth of about 8 m for a total sea bed area of approximately 2800 m².

Except for the collections described below, the *Aplysia* populations in these areas were protected from human disturbance.

Collections were made once each month at each site, beginning in July of 1972 at Site 1, and in June of 1973 at Site 2. A monthly collection at each site was continued through August of 1975. Each month, weather permitting, from two to five divers using SCUBA covered the bottom in a zigzag pattern between the depths of 1 and 8 m along the length of the study area (depths were approximate as indicated by divers' depth gauges). Because most specimens of *Aplysia* were found in water shallower than 5 m, on some dives the area below this depth was not searched. Each specimen of *Aplysia* encountered was collected. Divers rarely found animals weighing less than 20 g due to the searching techniques used, and the tendency of smaller animals to be camouflaged amidst algal fronds. The data on abundance and weight presented in this paper therefore exclude all newly metamorphosed specimens and most juveniles of less than 20 g.

From July of 1972 through March of 1973, the presence or absence of copulating individuals was noted during the monthly collections. From April 1973 through the end of the study period, additional data were collected on underwater slates during the dives. These included the number of animals paired or aggregated, and in many cases the number of animals in individual aggregations. Paired animals were usually copulating and were always in physical contact with one another. An aggregation was defined as a group of three or more specimens of *Aplysia* in physical contact with one another. Such groups generally included individuals engaged in egg-laying, copulation, or both. At any given time the aggregation could include (or on rare occasions be entirely composed of) animals not engaged in either of these activities (see also Kupfermann and Carew, 1974).

After collection, the animals were brought into the laboratory where each was weighed (after being placed on a paper towel for a few seconds to drain off

excess water). Beginning in August 1973, each animal was tagged with a one-half inch Floy Tag (Floy Tag and Manufacturing, Seattle, WA), colored with waterproof marker and stamped with an identifying number or letter. A tagging gun was used to insert the tag through one of the parapodia. While in the laboratory, the animals were housed in aquaria supplied with a continuous flow of fresh sea water within 1°C of ambient ocean temperature. After being tagged and weighed, the animals were returned to the study site, usually within 24 hr of the time of capture.

Histological examination of gonads from *Aplysia* was performed each month from October 1973 through September of 1975. To assure a supply of gonads throughout the year and to conserve local populations, gonads were obtained from specimens of *Aplysia* sacrificed for neurophysiological research at the California Institute of Technology in Pasadena, California. These animals were obtained (through Pacific Biomarine Supply Co.) from Palos Verdes on the California coast about 25 km north of Catalina Island. These were maintained in a recirculating sea water system without feeding on a regular schedule of 12 hr of darkness alternating with 12 hr of light. The temperature was maintained at a constant 14°C .

The animals whose gonads were examined had been maintained under these conditions for an average of 11 days. Each animal was weighed after removal of excess water as described above. A piece of gonad (3 to 5 mm^2) was removed (preliminary measurements had shown that average oocyte diameter is uniform throughout the gonad) and fixed in Bouin's solution made with sea water. The tissue was then dehydrated, cleared, and embedded in Paraplast. Non-serial (usually every tenth section was retained) 5-mm sections were stained using Cason's modification of the Mallory-Heidenhain stain (Humason, 1967). The stained sections were examined microscopically, and oocyte diameter measured with an ocular micrometer. To establish a standard point of reference for measurement, only oocytes in which nucleoli were visible were measured. According to Thompson and Bebbington (1969), the nucleolus is prominent until the *Aplysia* oocyte has completely matured, at which time it disappears. Thus a slight bias against very large oocytes may be introduced by this method. The average diameter of the first 50 oocytes in which the nucleolus was visible was determined. Fixation seemed to have little effect on oocyte size, as the diameter of the largest oocytes measured ($80\text{ }\mu\text{m}$) was approximately the same as a newly laid egg. In extremely immature gonads, oocytes were small and difficult to measure with certainty. Based on initial measurements, an average value of $7\text{ }\mu\text{m}$ was assigned to the oocytes of such immature gonads. The number of animals sampled each month ranged from 5 to 20, but in 19 of the 24 months, ten or more animals were examined.

RESULTS

Seasonal variation in weight

Figure 1 shows size-frequency distributions of all collections at Site 1 in which more than six animals were found. Presentation of the data in this form

reveals some trends as well as the considerable variability in population structure from one year to the next. The tendency of small animals to appear in the winter and spring can be seen in 1973 and 1975. An increase in overall size during late spring and summer is evident in 1974 and 1975. Unpredictable fluctuations in size and numbers also occurred. In late winter and early spring of 1973, juveniles appeared as expected, and had begun to increase in size by May. In June, however, the population inexplicably disappeared and remained absent for the remainder of the summer and fall. A few animals appeared in December, and specimens of *Aplysia* in a range of sizes were present through the spring of 1974. The sizable component of small animals present in the spring of the preceding and succeeding years was absent.

Figure 2, which shows the trends apparent over the three-year sampling period, was generated by combining the data for each month throughout the sampling period. Each site is graphed separately, with each point representing the weighted average of the collections made during that month. Seasonal differences in the size of the animals are apparent. Highest weights were recorded during the summer months of June through August, while weights were generally lowest between February and May. There is also a significant difference in the weights of the populations found at the two study sites. These were compared by performing a

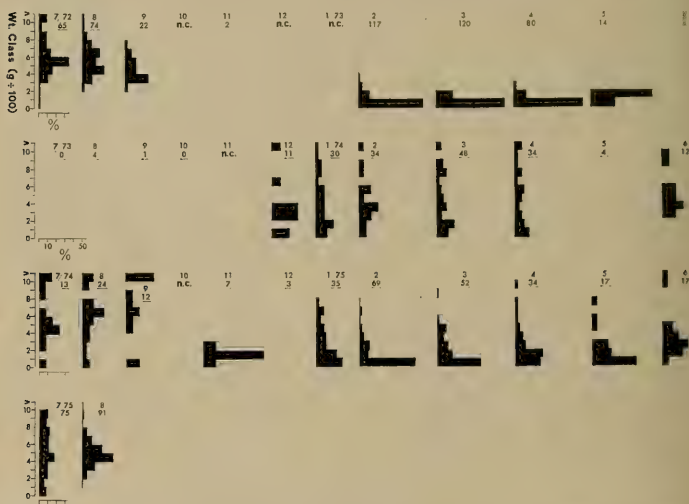


FIGURE 1. Size-frequency distribution of all collections at Site 1 in which more than six animals were found. The width of the figure corresponds to the percentage of animals found in each size class. The uppermost number is the date, while the underlined number immediately below it shows the number of specimens of *A. californica* collected that month. N.C.: no collection.

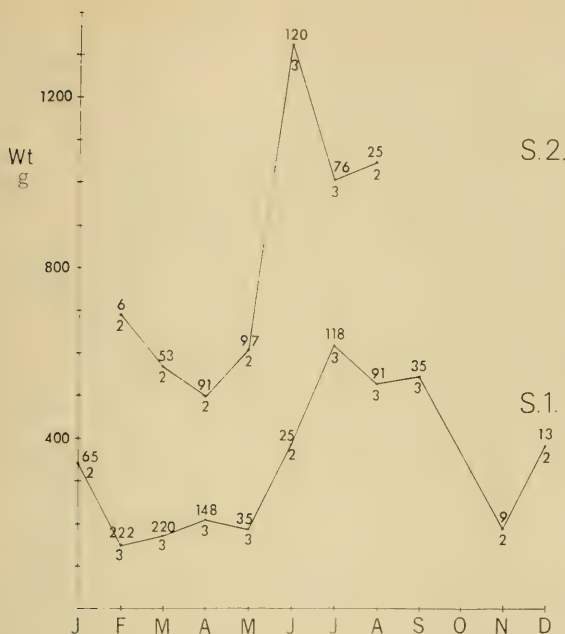


FIGURE 2. Average weights of specimens of *A. californica* collected at each study site, plotted by month. S. 1: Site 1; S. 2: Site 2. The upper of the two numbers associated with each data point is the total number of animals collected during that particular month throughout the study period. The lower number is the number of years in which samples were collected during that month. Only months in which six or more specimens of *A. californica* were collected are graphed.

paired *t*-test on the average weights for the ten individual months in which more than six animals were collected at each site (two-sided paired *t*-test; $t = 3.6$, $P < 0.01$). The two study sites differed in the availability of food algae readily consumed by *Aplysia*. Site 1 was dominated by beds of the surfgrass *Phyllospadix* to a depth of about 3 m. This plant is not normally consumed by *Aplysia* (Winkler and Dawson, 1963; personal observation). Below 3 m, the giant kelp *Macrocystis* predominated. Numerous other algal species were present on the rocky substrate including several which have been reported to serve as food for *Aplysia* (Leighton, 1966). Of these, only two were readily consumed under laboratory conditions, *Laurencia* and *Plocamium*. Neither of these algae was abundant at Site 1. Site 2 supported (among many other algal species) large beds of *Plocamium* between the depths of 1 and 4 m. Below this depth, *Macrocystis* predominated.



FIGURE 3. Graph of data from Table I. Changes in weight are expressed as percentages of initial body weight and plotted according to month. Data from both study sites collected throughout the tagging program are presented. Only months in which five or more animals were recaptured are graphed.

The average weights of animals used at Caltech also show seasonal variation, in spite of attempts by Pacific Biomarine Supply Co. to provide animals of roughly uniform size. Figure 6 shows that animals were smallest during late winter and early spring, while maximum weights were reached during the summer and fall. This parallels the trend seen in the populations studied on Catalina Island.

Tagging and recapture

A total of 154 specimens of *Aplysia* were recaptured between June of 1973, when the tagging program was initiated, and August of 1975, when the study was completed. Altogether, 728 animals were tagged during this period. Of those recaptured, 101 had been tagged the previous month. Only these were used to generate Figure 3, to best illustrate the growth trends typical of individual months. In Figure 3, field weight changes have been expressed as percentages of beginning body weight. Data have been combined for animals collected at both study sites, and for the same month throughout the tagging period. Only months in which five or more individuals were recaptured are graphed. Since few animals were found in winter (Fig. 4) there were few recaptures between October

and January. Again, strong seasonal trends are evident, with the maximum percent increase occurring in late winter and early spring when the animals are relatively small, and decreasing as the average weight of the population increases. A net loss of weight occurred during August and September. Table I shows the number of recaptured animals which had gained or lost weight during each month of the year. Weight loss was almost never observed between January and June, but after June, the majority of animals recaptured had lost weight.

In some instances, the same individual was recaptured more than once, providing information on the weight changes which individuals experience with age and season. These cases reveal that this species is capable of considerable growth during a single month. One animal from Site 2 gained 910 g between February and March of 1975, and an additional 1178 g between March and April of that year. Two animals which were captured first in the spring and later in the summer each showed an initial weight gain, followed by summer weight loss.

In this study, specimens of *Aplysia* were tagged with considerable success. Nine animals were recaptured after four or five months, indicating that some individuals spend a considerable time within a limited area. This species is capable of traveling relatively long distances, however. Kupfermann and Carew (1974) found that *A. californica* could easily cover a distance of 75 m (net) in a two-day period. Because many animals could have moved out of the study areas from one month to the next, recapture data from this study were not dependable for population size estimates.

Seasonal changes in abundance

It was noted during collections that the abundance of *Aplysia* seemed to vary seasonally, with a maximum during the spring, and a nearly total disappearance during the months of October and November. Abundance was estimated by determining the number of animals captured per unit effort, with one unit of effort defined as one diver-hour. The trend that emerges when data taken throughout the study are graphed by month is shown in Figure 4A. While few or no animals were found during regular monthly dives between October and December, juveniles ranging from 1 mm to 2 cm in length were found on *Plocamium*

TABLE I
Weight changes under field conditions.

Month	N	Initial Wt. (mean, g)	Wt. Change (mean, %)	No. gaining	No. losing
Jan	2	380	42	2	0
Feb	5	475	85	4	1
Mar	16	386	54	16	0
Apr	11	695	98	11	0
May	7	545	68	7	0
Jun	12	865	30	10	2
Jul	15	1060	35	7	8
Aug	31	644	-13	3	27
Sep	5	906	-11	2	3

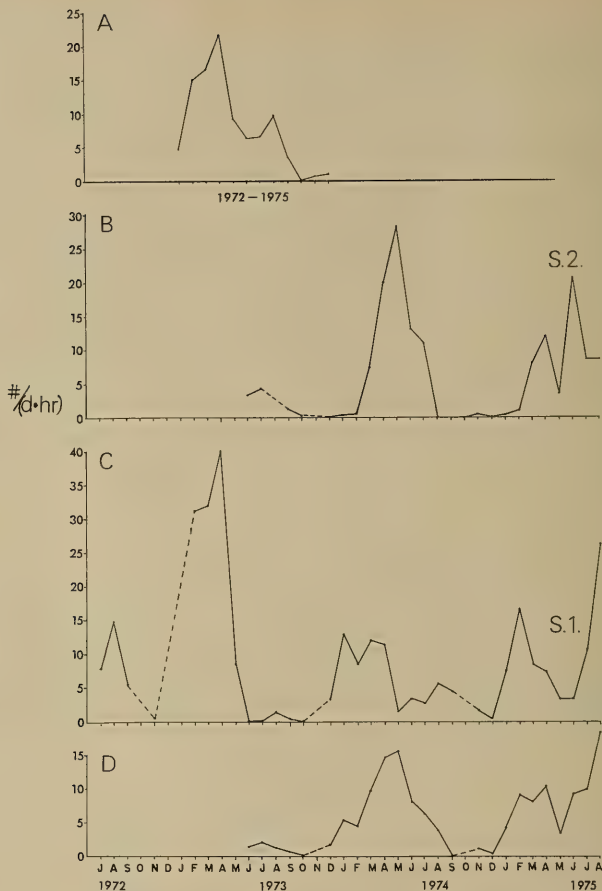


FIGURE 4. Abundance of animals expressed as *Aplysia* per diver-hour, plotted against the month. A: data from both study sites over the entire collection period are combined in a single "composite year". B: abundance at Site 2 plotted against actual time. C: abundance at Site 1 plotted against actual time. D: average abundances for both sites graphed for that period during which both sites were sampled. Dotted lines indicate that one or more collections were missed during the period covered.

and *Laurencia* brought into the laboratory. Juveniles very similar in color to the red algae were found hidden among the fronds, and these small animals seemed to be especially abundant during fall, winter and early spring.

Although distinct trends emerged from the total data collected (Figure 4A), Figures 4B, C and D show the considerable variability of the two sites from year to year. For example, collections at Site 1 during June, July and August 1973 produced a total of five individuals, while collections during the same months of 1975 produced 183 animals (including 25 recaptures).

A predictable occurrence at both sites was the nearly total disappearance of animals in the fall (October through December). Many individuals collected during this period exhibited symptoms of physical deterioration. These included erosion of the free edges of the parapodia and loss of pigment resulting in pale patches on the body. Such animals frequently died in captivity, an unusual occurrence at other times of the year. Few dead animals were found in the field at any time of the year.

Reproductive activity

When collections were initiated at Site 1 in July of 1972, copulation was already in progress. The following year, no copulation or egg-laying was observed there due to the disappearance of the population in June (May was the last successful collection in 1973 at Site 1, and no mating was observed at that time.) Collections at Site 2 began in June 1973, at which time copulation was occurring. In 1974, a few large animals were first seen mating in April at Site 1, and in May at Site 2. In 1975, again a few large animals were seen mating at Site 2 as early as April, but at Site 1, copulation and egg-laying were not observed until July, at which time many animals were participating.

Data on the percentage of animals aggregated, and the number of animals making up aggregations, are plotted in Figure 5. In April and May, the percentage of animals observed in pairs and aggregations was small, but increased to 80% or more during the months of July and August (Fig. 5A). Fifty-three aggregations are plotted according to the number of animals participating (Fig. 5B), which ranged from 2 to 19. Pairs of animals were observed more frequently than any other size grouping. Since *Aplysia* mates in chains of indefinite length, a pair of animals may be considered the shortest possible mating chain.

Seasonal changes in oocyte diameter

The data graphed in Figure 6 show the changes in oocyte diameter which underlie seasonal breeding in *A. californica*. Most animals collected in winter (January through March) have extremely small gonads containing few recognizable oocytes, these averaging about 7 μ m in diameter. In April and May, the gonad is characterized by a mixture of oocytes of all sizes, ranging up to 80 μ m, the approximate diameter of a newly laid ovum. The proportion of large oocytes increases with time, until, by the months of September, October, and November, mature animals show few of the small oocytes which predominate in the spring. The low standard error in average oocyte diameter in animals collected between January and October indicates that oocyte maturation is relatively synchronized. In November and

December, the standard error increases due to the inclusion of some sexually immature animals. It should be noted that, during the winter months, size is not an accurate indicator of sexual maturity. Sexually immature specimens of up to 600 g have been found at this time.

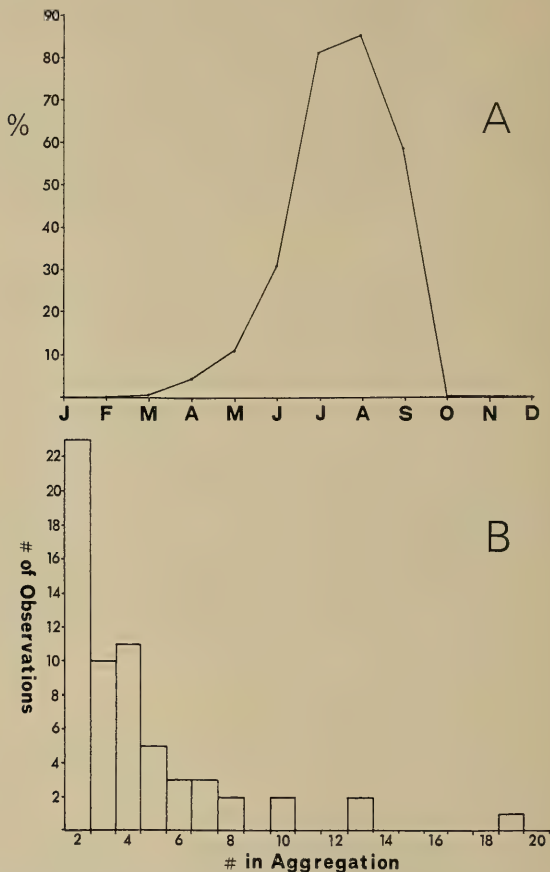


FIGURE 5. A: percentage of the total number of specimens of *Aplysia* captured each month that were paired or aggregated. Data from both study sites throughout the collection period are combined. B: frequency of observation of aggregation plotted against aggregation size.

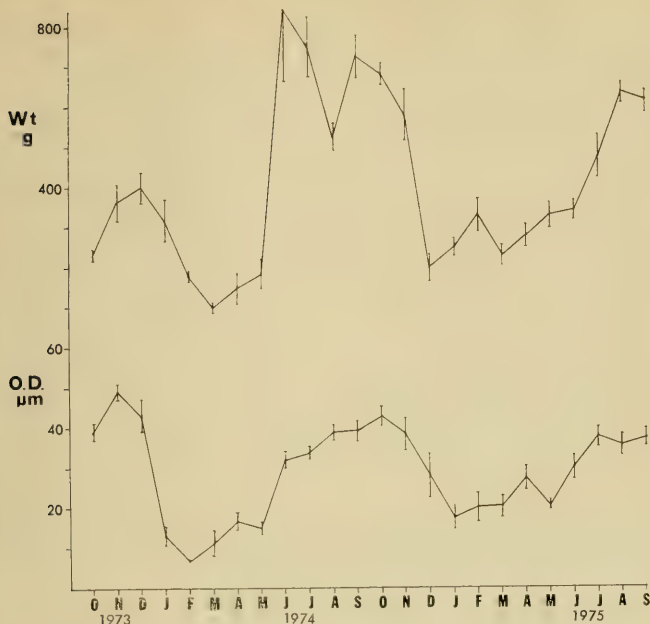


FIGURE 6. Mean weights (upper line) and oocyte diameters (lower line) of specimens of *Aplysia* plotted against time. These animals were collected at Palos Verdes, California, by Pacific Biomarine Supply Co. Most data points represent at least 10 individuals (see text). These were sacrificed throughout the month indicated, and collected an average of 11 days prior to being killed. Bars indicated standard error of the mean.

DISCUSSION

Data presented in this paper support the hypothesis that *Aplysia* have a life expectancy of approximately one year, and a single extended period of copulation and egg laying which begins late in spring and ends in the fall with the death of the sexually mature population. Although migration into deeper water and reappearance in the spring cannot be entirely ruled out, there is presently no evidence in support of this hypothesis. Animals found in the spring tend to be smaller and more abundant than those found later in the year. They appear healthy and live for long periods under laboratory conditions, in contrast to animals obtained in the fall whose skin is often eroded and who usually die after a short period of captivity.

The extended period of egg production might be expected to result in asynchrony with respect to size and sexual maturity within the population. However, there

is evidence that increasing water temperature in the spring provides a synchronizing cue for the initiation of gonadal development (Audesirk, 1976; Smith and Carefoot, 1967). Differences in survival and growth rate during different seasons might also result in greater uniformity in size than would be predicted from the long period of egg-laying. Further study is required to test these hypotheses. The disappearance of nearly all animals larger than 20 g at Catalina Island as early as October is in contrast to the situation at Palos Verdes where sexually mature specimens are found until December. A comparison of environmental factors including water temperature, food availability, and wave exposure between the two locations might provide clues to this discrepancy.

The mean weights of the populations at Palos Verdes and Catalina show pronounced seasonal differences consistent with an annual species (Figures 1, 2, and 6). On Catalina, small specimens of *Aplysia*, presumably offspring of the previous reproductive season, make their appearance between January and May. Maximum size is reached in June or July, and shows a decrease in August and September. In October, November and December, few animals are found (Fig. 4). Although trends in mean weight were roughly parallel for the two Catalina Island populations sampled, a consistent and significant size difference was observed (Fig. 2). The apparent explanation for this is the relative availability of food algae at the two sites. At Site 1, no algal species known to be readily consumed by *Aplysia* under laboratory conditions was found in abundance. In contrast, Site 2 included large beds of the red alga *Plocamium* which *A. californica* will eagerly consume. In his study of *A. punctata*, Carefoot (1967a) found a strong correlation between the size of the animals and the abundance of *Plocamium*. In a comparison of eight algal species consumed by *A. punctata*, *Plocamium* was preferred over six other species, and supported most rapid growth (Carefoot, 1967b).

Data from tagging and recapture (Fig. 3; Table I) indicate that, at Catalina Island, *A. californica* experiences fastest growth between February and April, prior to the onset of breeding. The animal's capacity for rapid growth explains how some individuals may achieve sizes in excess of 3000 g in a single year. Growth slows as breeding intensity increases, and the average size reaches a peak in June or July. In August, when breeding is at its most intense (as indicated by the percentage of animals in pairs or aggregations in Fig. 5), a net loss of weight occurs. This loss is possibly attributable to two major causes. The first is loss of foraging time due to time spent in reproductive activities. Although the percentage of time spent in breeding has never been quantified, field observations indicate that individuals could remain in aggregations for a day or longer. Kupfermann and Carew (1974) tagged the five animals at one aggregation site and, during four visits during the next five days, always observed one or more tagged animals at the site. The animals were not individually identified, and some had certainly departed and returned, but others may have remained. Aggregations and pairs of individuals appear almost exclusively during months when copulation is observed. It is hypothesized that the animals in aggregations which are not engaged in egg-laying or copulation at any given time may have recently completed one or more of these activities, or may be about to engage in them. Further study is needed to test this hypothesis. During the present study, at Site 1 nearly all the mating aggregations were discovered in dense beds of the surfgrass *Phyllo-*

spadix, whose closely spaced blades seem to present an ideal anchoring site for the tangled egg masses. Since they do not consume this plant, the animals at Site 1 probably did not eat during the time spent in aggregations. Kupfermann and Carew (1974) also never observed feeding by animals in breeding aggregations.

The second major factor contributing to weight loss is the massive egg production by these hermaphrodites (MacGinitie, 1934) which requires large energy expenditures.

Although seasonal trends in abundance are evident, these are superimposed upon dramatic fluctuations from one year to the next. Beeman (1977) noted that opisthobranch populations "tend to be erratic and sporadically explosive," a generalization with some applicability to *Aplysia*. Eales (1921) and Carefoot (1967a) reported similar fluctuations in populations of *A. punctata*.

Like *A. punctata*, which is found dead or dying in the field between September and December (Carefoot, 1967a), *A. californica* shows physical deterioration manifested externally as tissue erosion during the same months. A related phenomenon was reported by Lickey, Wozniac, Block, Hudson, and Augter (1977), who noted that specimens of *A. californica* in the laboratory seldom die between December and July, but rarely survive more than a month between August and November. For unknown reasons, dead animals were rarely found in the field at Catalina Island. In the laboratory, however, death was common during these months.

The brief life cycle of *A. californica* and the dramatic changes in its physiology and behavior as it progresses from sexual immaturity through the reproductive state to senescence have important implications for investigations of *Aplysia* neurobiology. Changes in neurohormones with respect to season have already been noted (Strummwasser, Jacklet, and Alvarez, 1969). The formation of breeding aggregations, copulation, and egg-laying are dramatic behavioral changes correlated with sexual maturity. The imminent death of mature animals obtained in the fall must also be taken into account during investigations of *Aplysia* behavior and nervous system function.

SUMMARY

1. Observations of field growth rates, reproductive activities, and abundance of *Aplysia californica* were made over a three-year period on Santa Catalina Island off southern California.

2. The mean weight of the population was found to vary with the location in which the animals were collected, presumably as a result of differing availability of food.

3. Seasonal weight differences were also apparent. In general, small specimens of *A. californica* appear between February and May. Mean weight reached a maximum between June and August. Considerable variability was encountered from year to year.

4. Tagging and recapture showed that growth rates reached a maximum in the spring just prior to breeding. The rate decreased thereafter until weight loss was experienced in August and September.

5. *A. californica* was usually most abundant in the spring, with numbers decreasing during the summer. The animals almost completely disappeared during the months of October, November, and December with the exception of extremely small specimens found on algae.

6. Breeding activity was occasionally observed as early as April and reached its greatest intensity during July and August when at least 80% of the animals collected were in breeding aggregations.

7. Histological examination of gonads showed maximum oocyte diameter between June and October, and minimum between January and March.

8. Data are consistent with an annual species whose extended summer breeding period is terminated by the death of mature individuals during the fall.

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SALT AND WATER BALANCE IN TWO MARINE SPIDER CRABS,
LIBINIA EMARGINATA AND *PUGETTIA PRODUCTA*. II.
APPARENT WATER PERMEABILITY

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In studies of salt and water balance, permeability is an important parameter since knowledge of it, together with a knowledge of the appropriate driving force, permits predictive statements concerning net fluxes. A complication in this matter is that permeability may vary. A reduction in tracer water exchange rate has been found in a number of osmoregulating decapod crustaceans transferred to a dilute medium (*Rithropanopeus harrisi*, Smith, 1967; Capen, 1972: *Carcinus maenas*, Smith, 1970; Berlind and Kamemoto, 1977: *Hemigrapsus nudus*, Smith and Rudy, 1972; *Palaemonetes pugio*, Rosejadi, Anderson, Petrocelli and Giam, 1976). However, not all osmoregulating decapods respond to a dilute salinity in this fashion (*Palaemonetes varians*, Parry, 1955: *Carcinus maenas*, Rudy, 1967: *Penaeus duorarum* and *Uca* spp., Hannan and Evans, 1973), nor does the same species necessarily respond in the same way under similar conditions. Also, there are alternative explanations for changes in water exchange other than a true change in permeability (Smith, 1970, 1976). However, the adaptive value of a reduction in water permeability when the osmotic gradient is increased is quite clear and, on the basis of the available evidence, a strong argument can be made in favor of changes in water permeability.

There is, perhaps, little reason to predict that an osmoconforming crab would respond to dilute salinity with a reduction in permeability; however, when specimens of *Libinia emarginata* were transferred from 100 to 80‰ sea water, there was a reduction of 30% in water exchange after the first hour (Cornell, 1973). It can be argued that a reduction in water permeability is also an adaptive response to dilute salinity in an osmoconformer, but the argument is open to question. In the present study, some aspects of apparent water permeability have been examined in the osmoconforming crabs *Libinia emarginata* and *Pugettia producta* and in the osmoregulating crab *Carcinus maenas*. An attempt is made to identify some factors which may be important in changes in water exchange rates.

MATERIALS AND METHODS

General procedures

Specimens of *Pugettia producta* were maintained at 10-12° C in filtered sea water (SW), about one crab per 4 liters, at the University of California, Berkeley, California. Specimens of *Carcinus maenas* and *Libinia emarginata* were maintained at 19 to 21° C in running SW at the Marine Biological Laboratory, Woods

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Hole, Massachusetts. The SW used at Berkeley was obtained from the Bodega Marine Laboratory, Bodega Bay, California and had an osmotic pressure of about 1015 milliosmols, while that at Woods Hole was about 945 milliosmols.

Exchange rates of tracer water were measured using D_2O according to the technique described by Welsh, Smith and Kammer (1968). Specimens of *Carcinus* and *Libinia* were immersed for 11 and 15 min, respectively, in aerated 3-liter solutions of sea water (SW) plus 2.0 to 2.5% D_2O maintained at constant temperature ($21 \pm 0.5^\circ C$) in a bath of running SW. Samples of blood were withdrawn by puncturing an arthroal membrane with a drawn-out Pasteur pipet.

Heart rate was determined by implanting chronic platinum electrodes which just penetrated the hypodermis over the region of the heart. The electrodes were cemented to the carapace with quick-setting ("five-minute") epoxy glue, after scraping away the epicuticle from the region where the bond was to be made. Electrodes were implanted at least 24 hr prior to an experiment.

Measurements of pressure changes caused by the beat of the scaphognathite were made with a Statham model P23BC pressure transducer connected by polyethylene tubing (P.E. 190, 0.047 inch = 1.2 mm inside diameter) to the cut-off tip of an 18-gauge hypodermic needle. A hole, positioned by external landmarks, was drilled through the carapace, and the needle tip was inserted through the hole and into the branchial chamber, just posterior to the scaphognathite. The needle tip was cemented in place with quick-setting epoxy. Measurements of heart and scaphognathite rates were recorded on a Grass Instrument Co. model 7a polygraph.

Isolated gills from *Libinia* were first flushed and then perfused with a solution containing inorganic ions in ratios given for *Cancer* perfusion fluid (Welsh, Smith and Kammer, 1968) and dextrose (1.5 mM). The solution was buffered to pH 7.2 with "Tris" (about 25 mM) and the final solution was adjusted to 945 milliosmols. A peristaltic pump, either a Buchler Instruments Polystatic Pump or a Sage model 375A, controlled the rate of perfusion and recirculated fluid between the gill and a small reservoir from which samples were taken. The total fluid volume of the system was about 1.5 to 2.0 ml. Polyethylene tubing was fitted to the afferent and efferent vessels and secured with silk thread. A small clamp, placed about 5 mm from where the tubing entered the gill, maintained the proper spatial relationship between the two tubes. A rod, attached to the clamp, suspended the preparation in a beaker containing 300 ml of aerated SW plus 3% D_2O maintained at $20 \pm 0.1^\circ C$. Constant stirring was provided by a magnetic stirrer.

A practical approach to estimates of apparent water permeability

Two different measurements of water permeability can be made, osmotic permeability and diffusive permeability.

Osmotic water permeability (P_{osm}) = $L_p RT / \bar{V}_w$, where L_p is the hydraulic conductivity, R is the gas constant, T is the absolute temperature, and $\bar{V}_w$ is the partial molar volume of water. L_p is a measurement of permeability and, simply stated, is a flux divided by a driving force. The advantage of converting L_p into P_{osm} is that by doing so, it is possible to compare P_{osm} with estimates of diffusive water permeability, since both can be expressed in the same units.

Unfortunately, the conditions under which L_p may be determined are incompatible with most whole-animal experiments. However, the concept of expressing a net flux divided by a driving force is useful and has been adopted here. The symbol L_p^* will be used to indicate a net flux divided by osmotic pressure and values will be expressed in $\mu\text{l}/(\text{g}\cdot\text{hr}\cdot\text{osmol})$. P_{osm}^* will be expressed in $\text{cm}^3/(\text{g}\cdot\text{sec})$ in keeping with the more conventional units [$\text{cm}^3/(\text{cm}^2\cdot\text{sec}) = \text{cm}/\text{sec}$]. When expressed in $\text{cm}^3/(\text{g}\cdot\text{sec})$, L_p^* is in the units of $\text{cm}^3/(\text{g}\cdot\text{sec}\cdot\text{atm})$, $R = 82.04 \text{ cm}^3/(\text{atm}\cdot\text{mole}\cdot\text{degree})$, T is absolute temperature in degrees K, $\bar{V}_w = 18 \text{ cm}^3/\text{mole}$. Since permeable surface area is unknown in a whole animal, weight has been substituted and allowances must be made for this factor in comparisons where large weight differences occur.

In a decapod L_p^* will reflect not only water permeability, but other factors as well. L_p^* only provides an adequate description of water movements across an ideal semi-permeable membrane which separates two solutions containing non-electrolytes. The characterization of a membrane which is permeable to solutes is more complex. When the solute is composed of univalent ions, the phenomenological approach based on irreversible thermodynamics requires the determination of six membrane coefficients (see review by House, 1974). Since water movement is influenced by the presence of permeant ions, L_p must be measured in the absence of an osmotic gradient and current flow, and in the presence of a driving force of hydrostatic pressure. Clearly, the measurement of net water fluxes under these conditions would be difficult in an intact decapod crustacean. It is believed, despite some objections, that the present approach provides a meaningful way of dealing with the problem of making measurements of "water permeability" in a whole animal.

Diffusive water permeability (P_d) = kv/A , where v is the volume of the water pool, A is surface area and k is a rate constant. In a well-stirred, two-compartment system in a steady state, the tracer concentration, $f(t)$, in the very small compartment which initially contains no tracer is given by $f(t) = C(1 - \exp -kt)$, where t is time, C is the concentration in the very large compartment, and k is a rate constant. The value of k is proportional to the diffusive water permeability, and in similar animals the constant is similar, so that k should provide a reasonable basis on which to make comparisons of permeability. In the present study, the results of the experiments with tracer water will be expressed in terms of the rate constant k in $(1/\text{hr})$, where $k = (1/t) \log_e [C/(C - f(t))]$. The symbol P_d^* in $\text{cm}^3/(\text{g}\cdot\text{sec})$ will indicate the analog to the more familiar P_d in cm/sec .

RESULTS

*Estimates of L_p^**

For an animal in a steady state, L_p^* may be estimated from the urine production rate and the osmotic difference between the blood and the medium. This approach is well suited to osmoregulators, but in an osmoconformer, the osmotic difference can be small (about 2 milliosmols in *Pugettia*) and thus subject to large errors in measurement. An alternative approach is to determine the rate of weight gain at time 0, when an animal is transferred to a dilute test medium.

TABLE I

Estimates of L_p^* in normal and nephropore-occluded specimens of *Libinia emarginata* and *Pugettia producta*. Analysis of covariance of the regressions of the transformed weights (see text) vs time indicates that there are significant differences between the initial rates of weight gain for normal specimens of *Libinia* ($N = 19$) and *Pugettia* ($N = 10$) ($P < 0.001$) and for nephropore-occluded specimens of *Libinia* ($N = 7$) and *Pugettia* ($N = 7$) ($P < 0.05$).

Animal	<i>Libinia</i>		<i>Pugettia</i>	
State of nephropores	Normal	Occluded	Normal	Occluded
Average weight (g)	152	144	121	117
Rate of weight gain at $t = 0$, expressed in volume [$\mu\text{l}/(\text{g}\cdot\text{hr})$]	30	33	70	67
Osmotic gradient at $t = 0$ (osmols)	0.189	0.189	0.203	0.203
L_p^* [$\mu\text{l}/(\text{g}\cdot\text{hr}\cdot\text{osmol})$]	159	174	345	330

At this moment, urine production is equal to that in the normal medium, the osmotic gradient is known, and the rate of weight change can be assumed to equal the net flux of water.

Empirically, it has been found that the equation $f(t) = Ct \exp -jt$ (where $f(t)$ is the weight at time t , C is a constant and j is a rate constant) provides a good description of weight changes in a number of decapods transferred to a dilute medium. At time 0, the rate of change of $f(t)$ can be determined from the first derivative and is simply C . Thus, by plotting the transformed weight data (transformed by $\log_e[(W_t/W_0) - 1](1/t)$, where t is time and W_t and W_0 are the weights at times t and 0, respectively) against time, the y intercept can be determined, and the value of C is equal to antilog_e of the y intercept. The resulting plots are seldom strictly linear, but they are sufficiently so that it is an easy task to estimate the intercept.

Using the above technique, the rates of weight gain were obtained from data on normal and nephropore-occluded animals (Cornell, 1976). Values for L_p^* were calculated from these rates and the changes in osmotic gradients. The results, presented in Table I, suggest that *Pugettia* is about twice as permeable to water as *Libinia*. The average for normal and nephropore-occluded animals is 166 and 338 $\mu\text{l}/(\text{g}\cdot\text{hr}\cdot\text{osmol})$ for *Libinia* and *Pugettia*, respectively. From these estimates, it is possible to calculate the expected osmotic difference necessary to produce an influx of water equal to the rate of normal urine production (2.1 and 2.5 $\mu\text{l}/(\text{g}\cdot\text{hr})$ for *Libinia* and *Pugettia*, respectively, Cornell, 1979); thus, $2.1/166 = 13$ milliosmols and $2.5/388 = 7.4$ milliosmols for *Libinia* and *Pugettia*, respectively. The estimate of 7.4 is greater than the observed value of 2 milliosmols in *Pugettia*. The osmotic difference between the blood and the medium is not known for *Libinia*; however, in both cases, these estimates are greater by about an order of magnitude than the colloid osmotic pressure (Mangum and Johansen, 1975). These discrepancies raise questions concerning the mechanism for the entry of water in an osmoconforming crab. One interpretation of the present results is that, whatever the mechanism, the driving force is equivalent to an osmotic pressure of between 7 and 13 milliosmols.

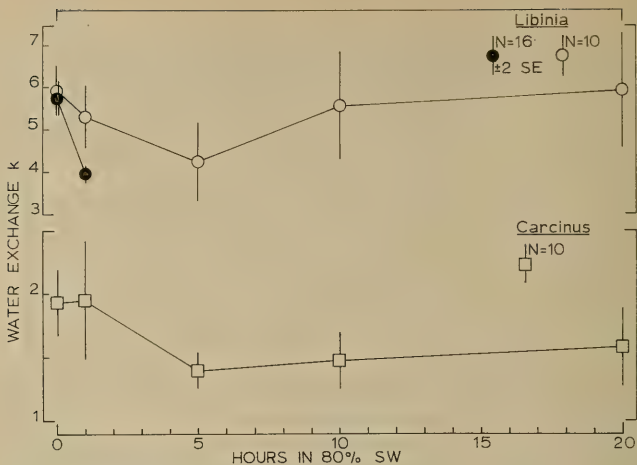


FIGURE 1. Changes in D_2O water exchange rates in specimens of *Libinia emarginata* and *Carcinus maenas* transferred from 100 to 80% SW, expressed as k in (1/hr), the hourly water exchange fraction. For specimens of *Libinia*, the results from two experiments are shown: the first, is indicated by the solid circles (average weight of crabs, 168 g); the second, is indicated by the open circles (220 g). For specimens of *Carcinus*, the results are indicated by the open squares (22 g).

I have calculated L_p^* for specimens of *Pugettia* during the first several hours after transfer to 80% SW. The calculations suggest that there is a reduction in L_p^* of about 60% during the first hour; however, this may be an artifact. The calculations, based on measurements which have relatively large variances (weight gain, urine production, and the osmotic gradient, Cornell 1976), become increasingly sensitive with time to errors in measurement. Since a plot of the logarithm of the osmotic gradient between the blood and the medium versus time is not significantly curvilinear, a large reduction seems unlikely. Perhaps the safest conclusion is that the data are inadequate to indicate whether or not a short-term change in L_p^* occurs.

Estimates of k

The results of two experiments in which specimens of *Libinia* were transferred from 100 to 80% SW are shown in Figure 1. For practical reasons, the points at time 0 were actually determined 24 hr prior to the transfer of animals to 80% SW. In the first experiment, k in (1/hr) was 5.68 for animals in SW and 3.99 for the same group after one hour in 80% SW, a reduction of 30% ($P < 0.001$, paired t -test). The second experiment is similar to the first, but was carried out for a longer period of time. In this experiment, k , measured after one hour of

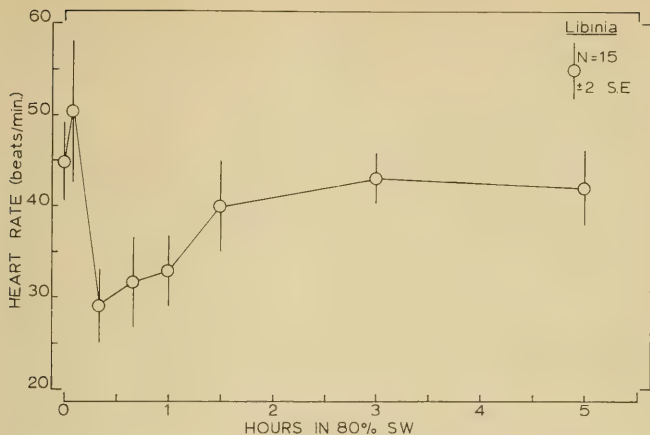


FIGURE 2. Changes in heart rate of specimens of *Libinia emarginata* transferred from 100 to 80% SW. Rates were determined from 2-min intervals (average weight of crabs, 150 g).

exposure to 80% SW, was not significantly different from k in 100% SW, but after five hours there was a significant decrease ($P < 0.005$, paired t -test). The reduction in k appears to be a transient response, since k returned to normal after ten hours. The cause of the difference in k between the two groups of animals is unknown.

While the reduction in k in *Libinia*, an osmoconforming crab, appears to be a transient response, long-term reductions have been reported for a number of osmoregulating crabs transferred to dilute salinities. The time course for this change has been reported for *Rithropanopeus* (Capen, 1972). In this crab, there is a reduction in k within the first hour of transfer from 75 to 10‰ SW. The initial phase of this reduction is not unlike that in *Libinia*, both in the initial rate of change and in the final extent of the change. It is of some interest to know if the time course in *Rithropanopeus* is similar to that in other osmoregulating crabs that have a reduced k in dilute media. To determine this, k was measured in specimens of *Carcinus maenas* transferred from 100 to 80‰ SW. The results of this experiment appear in Figure 1. For specimens of *Carcinus* in 100‰ SW, k was 1.94. After one hour of exposure to 80‰ SW, there was little change in k , but after five hours of exposure, the value had decreased by about 30% ($k = 1.41$, $P < 0.005$, paired t -test).

In the present study, k was determined in animals which were in a steady state, and in animals which were adapting to 80‰ SW and were not in a steady state. Therefore, it seems appropriate to ask if the reduction in k can be attributed solely to a change in state. The answer is probably "no." Two variables will be considered, surface area and volume of the water pool. The

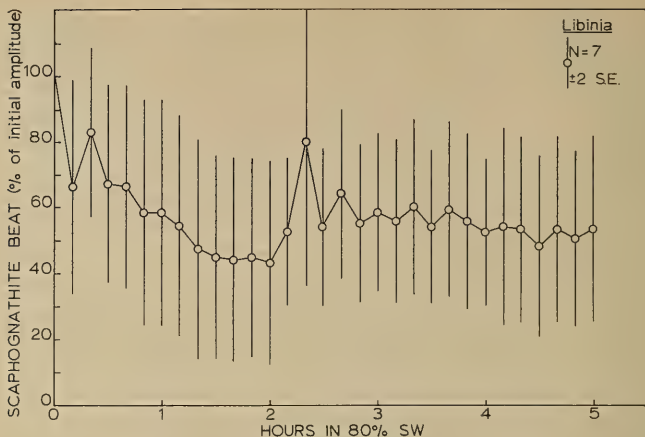


FIGURE 3. Changes in the amplitude of scaphognathite beat in specimens of *Libinia emarginata* transferred from 100 to 80% SW. Results are expressed as a percentage of the pressure developed by beats at time 0. The average amplitude of beat at time 0 was 0.7 cm H₂O and the average rate, 180/min (average weight of crabs, 163 g).

first, although difficult to evaluate, may be dismissed at the gross level on the grounds that whatever tendency for change exists is for an increase in surface area—a direction opposite to that necessary to account for the reduced k . The second may be dismissed on the grounds that, although an increase in volume occurs, and this would tend to reduce the value of k , the extent of the volume change (0.56% body weight in *Libinia* and much less in *Carcinus*) is insufficient to change the volume of the water pool (about 70% body weight) by an amount necessary to account for the reduction. The presence of more than one water compartment can also be considered; although this was not found in preliminary studies in *Libinia*, it has been reported for *Carcinus* (Rudy, 1967). Rudy found that the fast compartment was 60 to 80% of the total volume of exchangeable water; if all of the increase in volume occurred in this compartment, it would not account for the decrease in k .

Two possible mechanisms for changes in k

There is some similarity in the initial change in k in specimens of *Carcinus*, *Libinia* and *Rithropanopeus* transferred to a dilute salinity. Although this similarity may be superficial, there could be a common mechanism. There are, of course, many possible changes which could account for a reduction in k , but for present purposes, we will consider changes which occur in the unstirred layers at a liquid-solid interface. The concentration of a substance within an unstirred layer may be quite different from its concentration in the free medium, and under some condi-

TABLE II

Comparison of P_{osm}^* and P_d^* for some crabs. References are indicated by superscripts: a, this report; b, Born (1970); c, urine production from Shaw (1961), osmotic gradient from Smith (1970); d, urine production from Binns (1969), osmotic gradient from Smith (1970); e, Rudy (1967); f, Smith (1970).

Animal	Medium (% SW)	$P_{osm}^* = L_p^* \frac{RT}{V_w} \times 10^4$ [cm ³ /(g·sec)]	$P_d^* = \frac{kv}{g} \times 10^4$ [cm ³ /(g·sec)]
<i>Libinia emarginata</i>	100	28 ^a	11 ^a
<i>Pugettia producta</i>	100	56 ^a	36 ^b
<i>Carcinus maenas</i>	100-94		1.5 ^a , 4.6 ^f , 3.7 ^a
	80-70	5.3 ^c , 5.4 ^d	1.5 ^e , 5.3 ^f , 2.7 ^a
	50-40	4.3 ^c , 4.4 ^d	1.4 ^e , 3.9 ^f
	25-35	5.1 ^e , 7.5 ^d	3.4 ^f

tions, the thickness of this zone is an inverse function of the free stream velocity. Unstirred layers have a large influence on the rate of tracer water movement across very permeable membranes, since as permeability increases, diffusion through the unstirred layers becomes rate limiting.

For several crabs (*Rithropanopeus*, Capen, 1972; *Uca*, Hannan and Evans, 1973), it has been shown that 86 to 90% of tracer water exchange occurs in the gill chambers. Thus a change in blood circulation, or external irrigation of the gills could have a major influence on total water exchange. When specimens of *Libinia* were transferred to 80% SW there was a reduction in heart rate (Fig. 2). This could indicate a reduction in the blood circulation in the gills, and might account for the reduction in k . This idea received some support when it was found that water exchange was a function of the perfusion rate in isolated gills of *Libinia* maintained in SW: after 10 min of perfusion at 25, 47, and 90 ml/hr, the concentrations in the perfusion fluid, expressed as a percentage of the external medium, were 6.3 ± 2.84 (6), 14.2 ± 4.56 (6)*, and 18.2 ± 10.5 (5) % D₂O (mean $\pm$ SD (N), * = $P < 0.001$, paired t -test), respectively.

These is also evidence for a reduction in the rate of irrigation of the gills. In a typical 150-g specimen of *Libinia* in SW, the scaphognathite, or gill bailer, beats 150 to 200 times per minute. When this crab was transferred from 100 to 80% SW, there was a somewhat erratic, but definite reduction in the amplitude of the pressure pulse (Fig. 3), and a small tendency for a reduction in the rate of beat. Furthermore, in some animals, the scaphognathite appeared to stop for periods of several minutes. Most recordings were carried out for a 5-hour period, at which time the scaphognathite beat had not returned to normal. In one 15-hr recording, the scaphognathite beat returned to normal at about 9 hr. Considering the time courses, the change in the amplitude of the scaphognathite beat is better correlated with the change in k than the change in heart rate.

DISCUSSION

Comparative studies of decapod crustaceans, using tracer water (Rudy, 1967; Thompson, 1970; Hannan and Evans, 1973) and other techniques (Nagel, 1934; Gross, 1957; Herreid, 1969), have shown that there is a relationship between

"permeability" and habitat. In general, permeability decreases sequentially in animals from marine, brackish-water, and fresh-water habitats. Among closely related osmoconformers, permeability, as indicated by weight gain, seems to decrease with increasing euryhalinity (Davenport, 1972). *Libinia emarginata* and *Pugettia producta* provide an additional example of this type of relationship, since the lower apparent permeability of *Libinia* (Table II), can be correlated with its great salinity tolerance; *Libinia* can be adapted to 40% SW (Gilles, 1970) while *Pugettia*, to only 50 to 55% SW. These differences between *Libinia* and *Pugettia* are possibly related to the fact that environmental salinity fluctuations are greater on the east coast of the United States, where *Libinia* is found, than on the west coast of the United States, where *Pugettia* is found.

The relationship between tracer water exchange rate (k) and habitat reinforces the belief that k is a meaningful measure of permeability; however, some reservations about the meaning of small changes may be necessary. Generally among decapod crustaceans, changes in salinity have been found to affect k by about 20 to 40%. This change in k is small enough that factors other than permeability could be responsible. One such factor is a change in unstirred layers. In specimens of *Libinia* exposed to a dilute medium, the transient reduction in k , which initially resembles the long-term reduction in *Rithropanopeus harrisi* (Capen, 1972) and *Carcinus maenas* (this report), can be correlated with a reduction in the amplitude of the beat of the scaphognathite. In other studies, there are also indications of reductions in gill irrigation of crabs exposed to a dilute medium (*Libinia* and *Maja verrucosa*, King, 1965; *Carcinus maenas*, Hume and Berling, 1976). A temporary reduction in gill irrigation could provide a simple mechanism for short-term reductions in k and might confuse the issue of permeability changes since the scaphognathite is not only affected by salinity changes, but oxygen tension (McMahon and Wilkens, 1975) and tidal cycle (Arudpragasam and Naylor, 1964) as well.

Changes in blood flow could also affect k , but the evidence for this is not strong. Reductions in the heart rates of *Libinia* and *Carcinus* were thought by Cornell (1973, 1974) to provide a possible explanation for reductions in k ; however, further data make this possibility less likely. In addition, Hume and Berling (1976) found that the heart rate in *Carcinus* increases in dilute media. The cause of this discrepancy between the results of Hume and Berling and my own is not known.

It is generally believed that the effects of unstirred layers can be much greater on estimates of diffusive water permeability (P_d) than on osmotic water permeability (P_{osm}) (House, 1974). However, much of the discrepancy between these estimates may be eliminated by adequate stirring (Dainty and House, 1966). Thus, one possible way of evaluating the effects of unstirred layers comes from the comparison of P_{osm} and P_d .

It should be pointed out that it is difficult to justify the necessary assumptions in comparing P_{osm} and P_d in something as "simple" as an isolated sheet of epithelium, let alone an intact animal. But, we are guided by the words of House (1974, p. 341): "In view of the dubieties about errors in P_d and L_p values one has no proper right to compare them. Nevertheless, I shall do so." Table II shows P_{osm}^* and P_d^* for *Carcinus*, *Libinia* and *Pugettia*. In *Libinia* and *Pugettia*, P_{osm}^* was determined from the rate of weight gain in 80% SW at time 0 and

the osmotic gradient caused by the transfer from 100% SW. In *Carcinus*, P_{osm}^* was determined from published values of urine production rates, and osmotic gradients, for animals in a steady state in dilute SW. For estimates of P_d^* , it was assumed that the exchangeable water in all crabs was 70% body weight. P_{osm}^*/P_d^* is greater than unity in all cases. Interpreting these results is a matter for speculation, but I suggest that these data are consistent with the possibility that changes in unstirred layers could have a major influence on water exchange rates in all of these crabs since the ratio of P_{osm}^*/P_d^* does not differ greatly among *Carcinus*, *Libinia* and *Pugettia*.

Thus, a transient increase in the thickness of unstirred layers surrounding the gill might explain the transient reduction in k in a crab such as *Libinia*. Such a mechanism could also affect the initial phases of a long-term reduction in k in a crab such as *Carcinus*, but the evidence suggests that the maintenance of long-term reductions in k are the result of some other mechanism, which may be under hormonal control (see Tullis and Kamemoto, 1974, and Berlind and Kamemoto, 1977). The existence of this other mechanism is demonstrated by the fact that the isolated gills of *Callinectes sapidus* and *Cancer irroratus* from crabs adapted to 40% SW have a lower water exchange rate than do those from crabs adapted to 100% SW (Cantelmo, 1977).

In the strictest sense, it is possible to state that a change in water permeability has not been demonstrated beyond doubt in any decapod crustacean. But regardless, a process that can reduce the effect of an osmotic gradient is potentially of adaptive value. Thus, we should ask if a reduction in k reflects the action of such a process. A definitive answer to this question is not yet available for *Libinia*, *Pugettia*, and many other decapod crustaceans.

This paper constitutes part of a doctoral dissertation submitted to the Department of Zoology, University of California, Berkeley. It is a pleasure to thank Dr. Ralph Smith for his encouragement and guidance during the course of this work. I am grateful to Dr. Cadet Hand and the laboratory staff for their assistance and for the use of the facilities of the Bodega Marine Laboratory, Bodega Bay, California. I thank Dr. Robert Josephson for the opportunity to pursue my work while acting as a course assistant in the Experimental Invertebrate Zoology Course at the Marine Biological Laboratory, Woods Hole, Massachusetts. Financial assistance in the form of a one-year University Fellowship was greatly appreciated. My wife, Mary, deserves special thanks.

SUMMARY

1. Estimates of osmotic water permeability suggest that *Libinia emarginata* [$166 \mu\text{l}/(\text{g}\cdot\text{hr}\cdot\text{osmol})$] is less permeable to water than *Pugettia producta* [$338 \mu\text{l}/(\text{g}\cdot\text{hr}\cdot\text{osmol})$]. Data on tracer water exchange supports this conclusion and the differences in apparent permeability can be correlated with differences in habitat.

2. Short-term changes in D_2O water exchange were examined in specimens of *Carcinus maenas* and *Libinia emarginata*. When these crabs were transferred from 100 to 80% sea water, there was an initial reduction in k in (1/hr), the

hourly water exchange fraction, of from 1.9 to 1.4 and from 5.8 to 4.3 in *Carcinus* and *Libinia*, respectively. In both crabs, the initial response to the dilute medium is similar; however, in *Libinia*, the reduction in k is transient while in *Carcinus* it is a long-term response.

3. Estimates of osmotic (P_{osm}^*) and diffusive (P_d^*) water permeabilities for *Carcinus*, *Libinia* and *Pugettia* indicate that the ratio of P_{osm}^*/P_d^* is about 2, which suggests that unstirred layers could have a major influence on tracer water movement in all of these crabs. It is proposed that the initial changes in k , which occur during adaptation to a dilute medium, are at least partly the result of an increase in thickness of the unstirred layers surrounding the gills, caused by a reduction in the flow of medium through the gill chamber.

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UPTAKE OF AMINO ACIDS BY MARINE POLYCHAETES UNDER ANOXIC CONDITIONS

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Representative species of at least twelve different families of the class Polychaeta have served as experimental organisms in studies of transepidermal transport of small organic solutes in several laboratories. These studies include many published reports dealing with specific aspects of this phenomenon (reviewed by Stephens, 1972; Jørgensen, 1976) as well as unpublished work in this laboratory. Since marine annelids are often conspicuous members of the infauna, are easy to collect and maintain for brief periods in the laboratory, and live in habitats relatively rich in dissolved organic resources, they continue to be used for such work (Ahearn and Gomme, 1975; Rice and Chien, 1978; Stephens, 1975). Despite this attention, basic characteristics of this influx remain unexamined. We undertake to explore two aspects of influx of amino acids in two representative polychaetes.

First, the relation between influx of labeled substrates and net movement of substrate into or out of the animal is still in question. Stephens (1975) showed net influx of known amino acids in *Nereis diversicolor* and *Capitella* sp. using a fluorometric procedure to follow disappearance of added substrate. However, labeled substrates were not employed in this work. Details of the relation between influx of substrate followed radiochemically and net movement of substrate followed chemically have been described and analyzed for molluscs (Wright and Stephens, 1977, 1978; Crowe, Dickson, Otto, Colon and Farley, 1977) and for enchinoderms (Stephens, Volk, Wright and Backlund, 1978). The only report concerning annelids is that of Johannes and Webb (1970) in which they describe influx of ^{14}C -labeled glycine accompanied by net efflux of amino acids in *Clymenella*. Since many of the reports concerning influx of ^{14}C -labeled substrates overtly or tacitly assume that such influx represents net removal of substrate from the medium by the animal and draw conclusions on this basis, the subject merits investigation.

Second, infaunal polychaetes undergo periods of relative oxygen deprivation or anaerobiosis. If net influx of small organic molecules is to be invoked as a potential supplementary form of nutrition for these animals, the response of the process to relative or complete anoxia is important.

Stephens (1963) reported that influx of ^{14}C -labeled amino acids in *Clymenella* was unimpaired after long exposure to sea water through which gaseous N_2 was bubbled. However, no direct measurements of P_{O_2} were made. Several authors have used inhibitors of aerobic metabolism and reported partial inhibition of influx rates in some cases, and no clear response in other cases. None of this work includes independent criteria to assess inhibitor effects, and in most cases does not demonstrate reversibility of effects reported.

The present work reports the relation between influx and net flux of glycine in two genera of polychaetes. This relation is reported over a range of P_{O_2} including

anoxic conditions. The effects of anoxia are compared to effects of cyanide inhibition of aerobic metabolism.

MATERIALS AND METHODS

Specimens of *Marphysa sanguinea* (Eunicidae) were collected intertidally from Upper Newport Bay, Newport Beach, California. Animals were found in muddy sediment under and between intertidal rocks. Specimens of *Parcurelythoe californica* (Amphinomidae) were collected subtidally from a shallow lagoon at Point Mugu Naval Base near Port Hueneme, California. The animals occurred in sandy sediment just below mean low tide. Animals were maintained in the laboratory in aerated containers at 16° C. Specimens of both genera were selected in the wet weight range of 0.7 to 1.4 g for observations. Experiments were carried out at room temperature (23° C); animals were acclimated to the temperature change for several hours before use.

Influx of ^{14}C -glycine into animals was followed by periodic sampling of the medium in which animals were incubated. Experimental media were prepared from ^{14}C -gly (UL) at 20 to 40 $\mu\text{Ci/liter}$ plus sufficient ^{12}C -gly to achieve the desired chemical concentration. All media were prepared in artificial sea water (MBL) prepared according to Cavanaugh (1956) filtered through a Millipore filter (0.45- μm pore size). Duplicate 0.2-ml samples of medium were taken periodically, acidified to drive off CO_2 , and added to a toluene-based scintillation cocktail containing a detergent. Radioactivity was determined using a Beckman CPM-100 scintillation counter. Details of sampling protocol vary according to the experimental procedure.

Net change in ambient glycine concentration was followed using fluorescamine to determine changes in primary amines in solutions in which animals were incubated. The procedure has been described (Stephens, 1975; Stephens *et al.*, 1978). The reagent, fluorescamine, reacts with primary amines to produce a fluorescent product with an absorption maximum at 390 nm and emission peak at 480 nm. Fluorescence was measured using a Perkin-Elmer spectrophotofluorometer. Initially, fluorescence reflects glycine concentration since glycine is the only primary amine present in the medium. After incubation, fluorescence represents remaining glycine plus any primary amines which may be present as a result of efflux from the animal. Fluorescence is expressed in units of equivalent glycine concentration.

Influx and net flux of glycine under anaerobic conditions was measured as follows: Several hundred ml of MBL sea water was placed in a large culture flask with a port at the base in which an oxygen electrode (YSI) was mounted. Nitrogen gas was passed through acid pyrogallol and bubbled through the culture flask using a breaker stone. Oxygen content was monitored with the oxygen meter until anoxic conditions were achieved; approximately 5 min were required to reach the same oxygen reading as that obtained for sea water chemically deoxygenated with dithionite. Four samples of 50 ml each were then siphoned into four flasks, each containing one worm. Two of these flasks were placed in the N_2 gas train and maintained anoxic. The other two were reoxygenated using an air pump and served as controls. Worms were allowed to adapt to these conditions

TABLE I

Rates of uptake of glycine in specimens of *Pareurythoe* and *Marphysa* under aerobic and anaerobic conditions. In all cases, initial concentration of glycine was 20 μ M. Influx was calculated from the rate of depletion of ^{14} C-glycine from the medium; net influx refers to the rate of disappearance of total primary amines from the medium, and is expressed in terms of glycine-equivalents.

Experimental condition	Influx [moles $\times 10^{-7}/(\text{g} \cdot \text{hr})$]	Net influx [moles $\times 10^{-7}/(\text{g} \cdot \text{hr})$]
<i>Pareurythoe</i>		
Anaerobic	2.9	3.2
	3.0	3.2
	2.0	1.3
	2.5	2.3
	Average 2.6 ± 0.3 (s.d.)	2.5 ± 0.8
Aerobic	5.4	5.7
	4.6	5.2
	8.9	8.2
	7.8	8.0
	Average 6.7 ± 1.7	6.8 ± 1.3
<i>Marphysa</i>		
Anaerobic	2.2	—
	1.9	—
	1.2	1.2
	3.1	3.3
	Average 2.1 ± 0.7	2.3
Aerobic	7.4	—
	8.5	—
	4.9	4.9
	5.2	4.9
	Average 6.5 ± 1.5	4.9

for 15 to 30 min. Substrate (0.5 ml) was then added using a hypodermic syringe to initiate the experimental period. Samples were withdrawn periodically using a hypodermic syringe and were used for duplicate determinations of radioactivity and duplicate determinations of fluorescence at each time point.

Observations on influx and net flux of glycine in *Pareurythoe* were also carried out using a flow system. Filtered MBL sea water containing ^{14} C-labeled glycine (20 μ M) was placed in a flask to serve as a medium reservoir. A metering pump was used to produce a flow of medium through a chamber with sintered glass discs at either end (internal diameter = 16 mm, length = 75 mm, approximate volume = 15 ml). Medium from the reservoir was led to the chamber via a sampling port which permitted monitoring P_{O_2} as it passed into the chamber. The medium reservoir was initially deoxygenated and then reoxygenated to the desired level. By using a relatively large volume of medium in the reservoir with a small free surface, negligible drift in P_{O_2} was encountered during the course of observations. Flow rate was calculated to be sufficiently rapid that oxygen consumed by the worm would represent a depletion of 10% or less of the oxygen content of sea water in equilibrium with air. This represents a necessary compromise which allowed for a minimal flow rate sufficient to permit reliable determinations of the difference between inflow and outflow concentrations. Actual flow rates ranged from 3 to 6 ml/min depending on the weight of the worm. At

each level of P_{O_2} tested, medium was pumped through the chamber for 15 min prior to sampling. Samples were then taken each 5 min for the following 30 min and analyzed in duplicate for radioactivity as described. In some experiments the fluorescence at inflow and outflow ports was also determined.

In all cases where medium depletion was followed with time, a straight line was obtained when log radioactivity or log fluorescence was plotted against time, indicating that depletion followed first order exponential kinetics. Uptake rates are presented as moles glycine/(g wet weight·hour) from an ambient concentration of 20 μM . Data from the first hour of sampling was used to establish the rate constant. Uptake rates obtained using the flow system were calculated from the average difference between inflow and outflow samples as described and are expressed in the same units.

RESULTS

Table I presents rates of influx and net rates of influx for *Paracurthyoe* and *Marphysa* under anaerobic and aerobic conditions. There is no significant difference

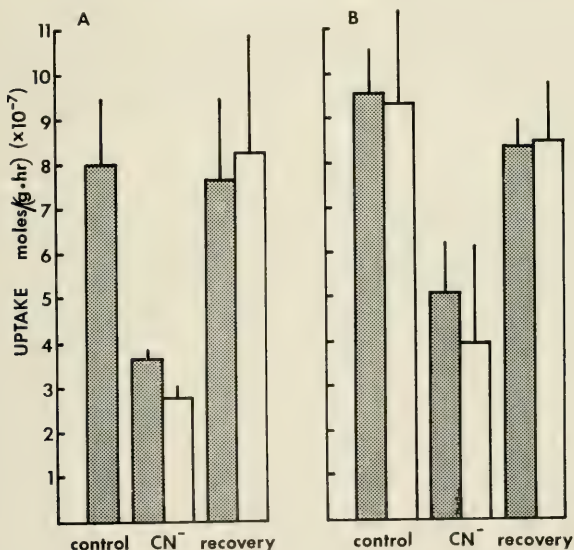


FIGURE 1. Effect of cyanide on the influx and net influx of glycine into A) *Marphysa* and B) *Paracurthyoe*. Influx (shaded bars) was calculated from the rate of depletion of ^{14}C -glycine from the experimental medium; net influx (open bars) was determined from the rate of depletion of total primary amines. In all cases the initial concentration of glycine was 20 μM ; the concentration of KCN was 2 mM. Bars represent ± 1 standard deviation.

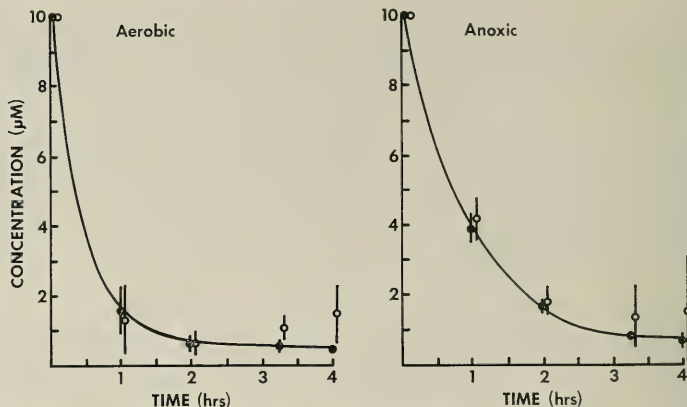


FIGURE 2. Effect of anoxia on the time course of influx and net influx of glycine into *Pareurythoe*. Solid circles represent measured levels of ^{14}C -glycine; open circles are concentrations of total primary amines expressed as equivalent glycine concentration. Anoxic conditions were produced by bubbling N_2 through experimental media containing 2 mM KCN. Each point is the mean of three separate determinations with individual worms; bars represent ± 1 s.d..

between rates of influx (^{14}C depletion) and net influx (decrease in primary amines). Hence there is a net transfer of substrate from the medium to the animal at a rate accurately estimated by either procedure. Rates of uptake under anaerobic conditions are reduced to levels 32 to 46% of aerobic rates.

Figure 1 presents data for influx and net influx of glycine in the presence and absence of 2 mM KCN. Oxygen consumption for three individuals of *Marphysa* was measured using an oxygen electrode. Exposure to 2 mM KCN for 30 min inhibited oxygen consumption to 13% of control values. Normal rates of oxygen consumption were restored when animals were permitted to recover for 24 hr. Influx and net flux of glycine into three individuals of each species before, during and after exposure to 2 mM KCN was measured on two separate occasions. Influx and net flux were reduced in the presence of cyanide to levels essentially the same as those observed under anaerobiosis (40 and 46% of control values). Recovery was complete.

Figure 2 presents data for *Pareurythoe* showing the time course of influx of ^{14}C -glycine and net flux of total primary amines under aerobic and anoxic conditions. The data for each experimental condition are mean values of separate studies on three worms. Anoxic conditions were produced by bubbling N_2 through experimental media also containing 2 mM KCN. Similar results were obtained using *Marphysa*. Note the close correspondence between influx as determined by depletion of radioactivity and net disappearance of substrate as indicated by determination of fluorescence. This is the case under both sets of conditions. In

both cases, determinations of fluorescence begin to diverge from determinations of radioactivity after two hours. In both cases, radioactivity does not continue to decline indefinitely. This does not represent a limitation of the transport system since ^{14}C -glycine at concentrations of 2 to $4 \times 10^{-7} \text{ M}$ is removed exponentially by both species.

Figure 3 presents data for two individuals of *Paracurlythoe* in which influx and net influx of glycine was measured in the flow system. Error bars represent ± 1 standard deviation of the average difference of seven determinations of radioactivity or fluorescence at inflow and outflow ports. Actual differences range from 0.6 to 7% of inflow. Since P_{O_2} was measured at the inflow, the values are systematic overestimates of the average P_{O_2} experienced by the animal during the observation period. Six such experiments were performed. In all cases there was a sustained high rate of influx, essentially comparable to that observed in aerobic depletion experiments, until ambient P_{O_2} was reduced to approximately 10% to 20% of air saturation values. In some cases, there was some decrease with decreasing P_{O_2} prior to the more conspicuous decrease observed at very low P_{O_2} . The data are too variable to justify mathematical treatment but suffice to demonstrate that influx and net influx are not linearly related to P_{O_2} over this range of oxygen concentrations. In these observations, influx and net influx of glycine were inhibited to a greater extent under anoxic conditions than the inhibition

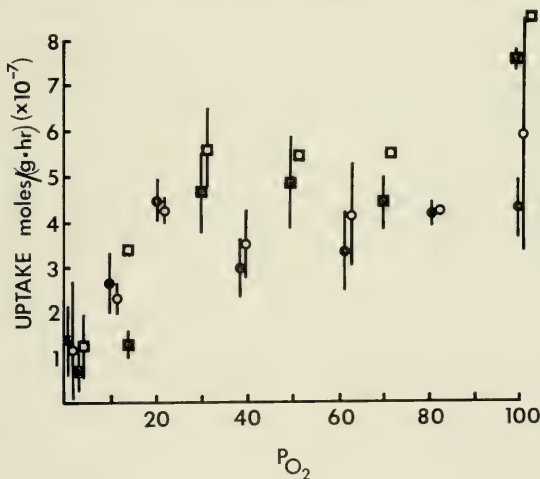


FIGURE 3. Effect of P_{O_2} on influx and net influx of glycine into *Paracurlythoe*. Circles and squares represent separate experiments on two individual worms. Closed symbols represent influx of ^{14}C -glycine; open symbols represent net influx of total primary amines. Bars are ± 1 s.d.; those cases in which open symbols have no error bars indicate that the variability was too large to include effectively in the figure.

TABLE II

Nutritional role of amino acid uptake in Pareurythoe and Marphysa: examination of critical parameters. See text for the basis of the calculations.

	<i>Pareurythoe</i>	<i>Marphysa</i>
Concentration of free amino acids in environment (μM)	123	131
Oxygen consumption [$\text{ml}/(\text{g}\cdot\text{hr})$]	0.13	0.15
Influx [$\text{moles} \times 10^{-7}/(\text{g}\cdot\text{hr})$] (cf. Table I, for 20 μM)	6.7	6.9
Contribution for 20 μM gly (μg)	50.5	44.6
% oxygen requirement	17.4	13.8
Glycine concentration for 100% oxygen requirement (μM)	115	145
Mixed amino acid concentration for 100% oxygen requirement (μM)	52	65

observed in depletion experiments. Anaerobic influx rates were approximately 15% to 20% of those observed at higher P_{O_2} .

DISCUSSION

There is no significant difference between rates of ^{14}C -glycine influx in *Marphysa* and *Pareurythoe* estimated from radiochemical measurements and rates of net influx of glycine estimated from fluorometric determinations from an ambient glycine concentration greater than 10 μM (Figure 2, Table I). There is a slow efflux of primary amines (fluorescamine-positive material) with time in both aerobic and anoxic conditions (Figure 2). These findings agree with similar studies of molluscs (Wright and Stephens, 1977, 1978) and echinoderms (Stephens *et al.*, 1978). In all of the soft-bodied marine invertebrates examined adequately, influx of ^{14}C -labeled amino acids reflects net influx quite accurately at concentrations greater than 10 μM ; however, this process is accompanied by a slow efflux of primary amines.

These results agree with the reports of Johannes, Coward and Webb (1969) and Johannes and Webb (1970) using the flatworm, *Bdelloura candida* and the polychaete, *Clymenella*. They supplied labeled substrates at low concentrations (0.6 μM and 1.0 μM in the studies cited) and observed a slow net efflux of amino acids with time at rates broadly comparable to those reported here and those reported by Stephens (1968). However, they interpreted their data as evidence for exchange diffusion and questioned the occurrence of net influx of substrate into marine invertebrates, suggesting that earlier literature might represent a misinterpretation of such exchange diffusion. However, exchange diffusion is clearly excluded as a mechanism of efflux by data showing that efflux is essentially independent of ambient concentrations, as appears to be the case in the examples previously cited. Beyond this, there is currently no evidence concerning the route or mechanism of efflux.

Table II presents measurements of naturally occurring primary amines in the immediate habitat of *Pareurythoe*. Data were obtained by expressing interstitial water from freshly collected sediment cores in the sandy subtidal habitat of *Pareurythoe*. Total free amino acids were estimated by the fluorescamine technique. Some of these data are reported in Stephens *et al.* (1978) where methodological details are presented. The samples ranged from 54 to 244 μM (average 123 ± 54

μM , $n = 13$). Free amino acids in the habitat of *Marphysa* were estimated in a similar fashion. However, it was not possible to obtain cores in the immediate area of the rocks where the animals were found. Therefore, cores were taken from otherwise comparable muddy sediment in immediately adjacent areas of Newport Bay. Average free amino acids ranged from 88 to 180 μM (average $131 \pm 35 \mu\text{M}$, $n = 7$). Table II also presents data for oxygen consumption of *Paracurthyoe* in the size range employed in these experiments [$0.13 \text{ ml}/(\text{g} \cdot \text{hr}) \pm 0.02$, $n = 6$]. For *Marphysa*, oxygen consumption was 0.145 ± 0.25 ($n = 8$). Table II also includes average rates of glycine influx (average of influx and net influx from Table I) for the two worms. Given these data, it is possible to calculate numbers to estimate the significance of amino acid influx as compared to metabolic requirements estimated from oxygen consumption. Complete oxidation of 1.0 mg of glycine requires 2.23 ml O_2 (STP). One can calculate the percentage contribution of glycine influx from an ambient concentration of 20 μM (where measurements were made) for the two cases. By making the assumption that influx at higher concentrations is linearly related to concentration, the glycine concentration in the medium which would provide sufficient reduced carbon to account for oxidative metabolism can be calculated. In fact, interstitial amino acids from the immediate habitat of *Paracurthyoe* have been identified chromatographically (Stephens *et al.*, 1978). Glycine is present as well as seven other identifiable amino acids accounting for approximately 85% of total primary amines as estimated by fluorescamine. Thus it is more realistic to use a conversion factor of 1 ml of O_2 required for complete oxidation of a mixture of amino acids provided one assumes that rates of glycine influx are comparable to influx rates for other amino acid substrates. The table also includes the ambient free amino acid concentration (based on these assumptions) required to provide reduced carbon equivalent to oxygen consumption.

These data and calculations indicate that influx of free amino acids from ambient solution occurs at rates that are of the same order of magnitude as the requirement for reduced carbon to sustain oxidative metabolism; *i.e.* the process of transepithelial influx represents a potentially important source of supplementary nutrition for these animals. This conclusion is based on the assumption that bulk concentrations of free amino acids measured in interstitial water of the sediment habitat are a reasonable estimate of concentrations available to the animals. There is no direct information on this point, though Stephens (1975) has provided evidence that irrigation activity of infaunal annelids may increase local free amino acid concentration in interstitial water.

These data and calculations are not relevant to conditions during periods of anaerobiosis which infaunal worms may undergo periodically. Under such circumstances, influx rates for glycine in *Paracurthyoe* and *Marphysa* decrease to 32 to 46% of aerobic rates. Requirements for reduced carbon will be considerably increased during periods of anaerobiosis. However, net influx continues from ambient concentrations in the range found in the habitat under anoxic conditions for up to 4 hr. Thus the process continues though its rate and overall contribution to metabolic requirements are both considerably reduced.

The presence of 2 mM KCN in the medium almost completely inhibits aerobic metabolism (oxygen consumption was reduced by 83%). This effect would be predicted based on the binding of CN^- to heme groups and the resulting interdiction

of electron transport. Thus the concentration used and the time of incubation employed were sufficient to force dependence on anaerobic metabolism. The resulting decrease in influx (40 to 45% of control values) agrees well with the decrease observed under anoxic conditions. Cyanide inhibition, both of influx and of oxygen consumption, were reversible; no mortality was encountered in these experiments. Thus this is additional evidence for the ability of these animals to tolerate periods of anaerobiosis. There is no evidence for a specific inhibitory effect of cyanide on the transepidermal transport system.

Mangum (1976) reviews respiratory adaptations of annelids. In her view irrigation of the burrow is one of the major adaptations to the sediment habitat in this group. Although data for *Marphysa* and *Pareurythoe* are not reported, it is reasonable to assume that they also irrigate their burrows. Mangum tabulates data comparing average P_{O_2} in the microhabitat (i.e. in the burrow) with P_{O_2} in the water column. The former ranges from 73 to 104 mm Hg when the overlying water column is essentially saturated. Thus the ability of the annelids examined in the present work to sustain normal rates of net influx at partial pressures of oxygen below saturation (Fig. 3) is of interest. There is little decline in influx rate until P_{O_2} drops below 20% of saturation. Since these estimates of the P_{O_2} actually encountered by the worms in the flow chamber are systematic overestimates (measured at the inflow end of the chamber), this suggests that the worms are functioning as aerobes at quite low P_{O_2} 's and hence at realistic levels of oxygen availability under normal circumstances. The depression of influx under anoxic conditions was greater in these experiments than was the case in the medium depletion experiments (15 to 20% rather than 32 to 46% of control values). The flow chamber was large in diameter compared to the worms. Under conditions of very low P_{O_2} , the worms typically coiled tightly. In contrast, medium depletion experiments were done with continuous gentle agitation by the gas stream (N_2 or air). It is possible that the greater depression of influx in the flow system is simply the result of this behavior and the reduction in surface exposed to the medium by the worms in the two situations.

The limitations of the use of oxygen consumption as a measure of metabolic requirements should be reemphasized. These limitations are based on the necessary assumption that metabolism is aerobic (clearly not always the case for annelids) and on the fact that requirements for organic material which must support growth and balance losses by other pathways do not enter the estimation process. It would be far more appropriate to undertake measurement of heat production (Pamatmat, 1978) and estimate minimum requirements on that basis; however, this has yet to be done for annelids. The calculations presented in Table II indicate that influx of naturally occurring free amino acids may provide a major input under aerobic circumstances. However, the significance of influx under anaerobic conditions will certainly be less, both by virtue of the reduction in rate and because of the increased requirements for substrate. In any case, the conclusion drawn from this work is that uptake of amino acids is a supplement. Both worms certainly have other feeding methods at their disposal.

The metabolism of facultative anaerobic invertebrates is complex and involves a variety of end products (de Zwaan, Kluytmans, and Zandee, 1976). Some of the pathways demonstrated produce amino acids such as alanine and succinate

and these compounds might be expected to accumulate under anoxic conditions (Hochachka, Fields, and Mustafa, 1973). Though such reactions may well be involved in facultative anaerobiosis in annelids, the resulting amino acids are not perceptively lost to the medium in *Marphysa* and *Pareurythoe*; rates of efflux of primary amines are not increased under anoxic conditions (Figure 2).

The extent to which the two species examined in this work actually experience anoxic periods is probable very different. *Marphysa* was collected from a population in the intertidal from an area characterized by fine-grained sediment of high organic content. At low tide, animals almost certainly experience anoxia. *Pareurythoe* was collected from a shallow sub-tidal population in an area of coarser sediment. It is not clear why *Pareurythoe* would ever experience anoxia so long as burrow irrigation continues. Both species tolerate anoxic periods well and behave similarly in the present observations. Mangum (1976) suggests that the circulatory system in annelids may play a more important role in acquisition of oxygen from the environment than in distribution to deeper tissues; in many species deeper tissues are virtually avascular. Possibly the widespread tolerance of anoxia among annelids may reflect the fact that deeper structures may be poorly supplied with oxygen even in aerobic conditions.

Finally, the relation of this work to earlier reports of influx of ^{14}C -labeled substrates into marine polychaetes should be mentioned. Since it is now quite simple to examine influx and net flux of amino acids, further work in this area should include such examination. However, the coupling of influx as estimated radiochemically and net influx examined chemically has now been reported for several major groups of marine invertebrates and in each case examined, influx has been shown to be a close estimate of net flux provided concentrations are realistic and initial rates are compared. It seems increasingly likely that the early reports can be accepted provisionally as providing evidence for the existence of transport systems capable of net transport of substrate from the medium into the animal. The potential contributions of such transport will depend then on environmental availability of substrate and will no doubt vary among species. The wide distribution of transepidermal transport systems among marine invertebrates indicates that further work is desirable to describe the extent to which energy flow by this pathway may contribute to the trophic organization of marine communities.

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SUMMARY

1. The effect of anoxia on influx and net flux of amino acids from dilute solutions into two species of marine polychaetes was studied.
2. Rates of influx and net flux correspond quite closely at ambient concentrations greater than $10\ \mu\text{M}$. Anoxic conditions, produced by incubating specimens of *Marphysa* and *Pareurythoe* in solutions containing $2\ \text{mM}$ KCN or through which N_2 was bubbled, did not affect the tight correspondence between influx and net flux, though rates were reduced by approximately 50%.
3. The effect of P_{O_2} on influx and net flux was examined using a continuous

flow system. Influx and net influx remained at control rates down to P_{O_2} 's 10 to 20% of air saturation values.

4. Comparisons of rates of net flux to measured values of O_2 consumption indicate that these animals can acquire sufficient reduced carbon to account for their oxidative needs if their surfaces are exposed to amino acid levels on the order of 50 to 65 μM .

5. Primary amines in the interstitial water of sediments in the immediate vicinity of populations of these worms averaged between 123 and 131 μM .

6. *Marphysa* and *Pareurythoe* live in habitats that are relatively rich in amino acids, and they possess transport systems capable of the net accumulation of these compounds at rates sufficient to provide a significant supplement to other forms of feeding. The uptake process continues during periods of anoxia, though its rate and overall contribution to metabolic requirements are reduced.

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PARTHENOGENESIS IN *COPTOPTERYX VIRIDIS*, GIGLIO TOS
(1915) (DYCTIOPTERA, MANTIDAE)

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The mantid *Coptopteryx viridis* has been studied for several years in our laboratory. As its behavior is similar to that of other species of the sub-order Mantodea, we considered the possible existence of a parthenogenetic reproduction mechanism. These animals are solitary and sedentary, and the female often kills the male before copulation takes place. The average adult life of the female is twice as long as that of the male (Guerrero, Maggese and Cukier, 1977).

Observations made by other workers indicate that *Brunneria borealis* reproduces exclusively by parthenogenesis (White, 1948a) and *Miomantis savignii* (Adair, 1925) reproduces both by parthenogenesis and by being fertilized. The present investigation was designed to determine the existence and type of parthenogenesis in *C. viridis*.

MATERIALS AND METHODS

Oothecas of *Coptopteryx viridis* were gathered in the vicinity of the slaughterhouse of Lisandro de la Torre in Buenos Aires city, and were kept in separate flasks. The nymphs which emerged from these oothecas were kept in individual cages for their whole life, at room light and temperature. The cages and the method of feeding were as described by Guerrero and De Carlo (1976).

Male nymphs of the 5th and 6th nymphal stages were employed for determination of the karyotype, and females of the 7th nymphal stage were used to corroborate the results. The testes and ovaries were immersed in an hypotonic solution of 0.7% Na citrate, fixed in Carnoy and stained with Giemsa.

Levan's classification was used to determine the karyogram where the centromeric index is $Ic = 100 \frac{s}{c}$ where s is short arm and c is whole length. Chromosomes with values of the index (Ic) of 50 are termed metacentric (type M); of from 50 to 37.5, metacentric (type m); 37.5 to 25, submetacentric (type Sm); 25 to 12.5, subelocentric (type st); 12.5 to 0, acrocentric (type t); and of 0 are termed telocentric (type T).

Study of the embryos involved fixation in Bouin, followed by manual elimination of the chorion, embedding in parafin, sectioning at 7μ thickness, and staining with hematoxylin-eosin.

RESULTS

Seven of the 13 females which arrived at the adult stage laid oothecas without fertilization. The number of oothecas laid by a female varied from 1 to 7, the variation depending on the time they lived as adults and on the time of the year in which they attained adulthood.

TABLE I

Animal No.	Date of birth	Date of molting to imago	Total nymphal life (days)	Time between the arrival to the adult stage and the first laying (days)	Number of oothecae per female	Interval between layings (days)	Total life time (days)	Date of death	Date of the last laying	Time between the last laying and death (days)
I (17)	22-Sep-75	26-Dec-75	95	80	2	9	203 108	12-Apr-76	24-Mar-76	19
IX (1)	15-Sep-75	22-Dec-75	98	77	1	—	178 80	11-Mar-76	8-Mar-76	3
X (3)	26-Sep-75	26-Dec-75	91	62	7	14 6 14 8 11 11	217 126	30-Apr-76	30-Apr-76	0
X (19)	3-Oct-75	26-Dec-75	84	77	3	19 7	199 115	19-Apr-76	7-Apr-76	12
X (22)	6-Oct-75	16-Jan-76	102	59	6	8 15 12 15 13	231 129	24-May-76	17-May-76	7
X (30)	13-Oct-75	19-Jan-76	98	69	1	—	167 69	29-Mar-76	29-Mar-76	0
X (37)	22-Oct-75	8-Mar-76	137	—	—	—	152 15	23-Mar-76	—	—
XII (82)	13-Oct-75	2-Mar-76	137	30	4	11 7 14	205 168	15-Aug-76	3-May-76	106

A total of 22 oothecas was laid by the 7 females (Table I). The time that elapsed between the achievement of the adult stage and the laying of the first ootheca varied from 30 to 80 days. The female that had laid her first ootheca on the 30th day had become an adult towards the end of the summer (2-Mar-76), whereas the one that delayed 80 days in laying her first ootheca had attained adulthood at the beginning of the summer (26-Dec-75). The rest of the females laid their first ootheca towards the end of the summer, having attained the adult stage at the beginning of that season. In general, the sooner they became adults the longer they delayed in laying their first ootheca.

The first ootheca was laid on 26-Feb-76 and the last one on 4-May-76, a span of two and a half months. The intervals of time between one laying and the next for all the females was from 6 to 19 days. The average intervals between successive layings per female varied between 9 and 13 days and the general average of intervals for all the oothecas laid by the 7 females was 11 days.

As a rule the females died immediately after their last laying, or a few days after it. The exception was one female (XII-82) which had arrived at the adult stage at the end of the summer, and lived 106 days after her last laying. As this laying took place at the beginning of May, it might be asked whether cessation of laying was due to low temperature, photoperiod or any other reason. Her ovaries were full of mature eggs.

The female IX-1 died 3 days after laying her last ootheca, with her ovaries full of mature eggs. They were not vestigial eggs since the last ootheca laid was of an enormous size. The female X-22 died 7 days after her last laying, and she had her ovaries full of mature eggs. The female X-30 laid her last ootheca on the day of her death, and her ovaries had no mature eggs. The female X-37 lived 15 days of adult life and her ovaries were full of mature eggs. She did not lay oothecas.

According to the present observations (Table I) it seems that the female would need a stimulus to start laying oothecas. This stimulus could be the presence of a male, if there was one; otherwise the female would wait until the last moment to lay her oothecas. Other probable stimuli could be environmental factors such as temperature and photoperiod. Once the process of laying started, it would not cease until the female died, or the environmental conditions turned unfavorable. It seems that the laying would depend on a certain period of the year more than on the chronotropic age of the individual. These data also indicate that about 30 days are needed to begin laying and that the yolk replacement is significant.

The next step was to wait until the oothecas hatched, since we were not sure that they contained viable eggs. The first nymph hatched 6 months and 18 days after laying, on 11-Oct-76 (Table II) and the last one on the 15-Jan-77.

According to our data for non-parthenogenetic populations, hatching never occurs after this date (Guerrero, Maggese, Cukier, 1977). The average interval between the laying of the ootheca and the first hatching was 7.5 months. From the 22 oothecas laid, 9 hatched nymphs, and 8 of the remaining 13 oothecas exhibited exposed cells that caused the eggs to dry. The number of nymphs eclosed per ootheca was very low, between 1 and 5. A total of 23 nymphs were hatched.

TABLE II

Individual N	Ootheca N	Number of hatched nymphs	Sex	Date of laying	Date of the first hatching	Time between laying and hatching	Date of the last hatched nymphs
X-22	1	2	female	15-Mar-76	3-Nov-76	7 months and 19 days	13-Dec-76
X-3	1	4	female	17-Mar-76	22-Nov-76	8 months and 8 days	25-Nov-76
X-22	2	3	female	23-Mar-76	11-Oct-76	6 months and 18 days	15-Jan-77
1-17	1	5	female	24-Mar-76	17-Nov-76	7 months and 24 days	13-Dec-76
X-3	2	1	female	31-Mar-76	7-Dec-76	8 months and 7 days	7-Dec-76
X-19	1	2	female	31-Mar-76	29-Nov-76	8 months	7-Dec-76
XII-82	1	1	female	3-May-76	29-Dec-76	7 months and 24 days	29-Dec-76
X-19	2	1	female	7-Apr-76	6-Dec-76	8 months	6-Dec-76
X-22	3	4	female	15-Apr-76	22-Oct-76	6 months and 3 days	13-Dec-76

The nymphs were all females, with a highly restricted viability since none of them passed the second nymphal stage (they lived between 0 and 14 days). Due to these results, the oothecas were kept for observation.

At the beginning of May, some of the parthenogenetic oothecas were opened and eggs in perfect state were found inside them. These eggs were fixed, embedded in paraffin and stained, revealing a very early stage of development. They presented a blastoderm along the surface with a ventral thickening that would represent the beginning of the development of the germinal band.

Oothecas collected from nature after the hatching period (3 months) also contained fresh eggs in the same stage of development. In these last oothecas it was found that several of those that had yielded a low progeny had a great number of empty chorions.

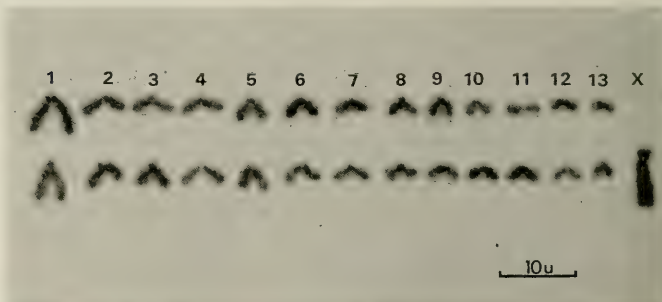


FIGURE 1. Diploid karyogram of *Coptopteryx viridis*. This was made from two metaphases of the second meiotic division.

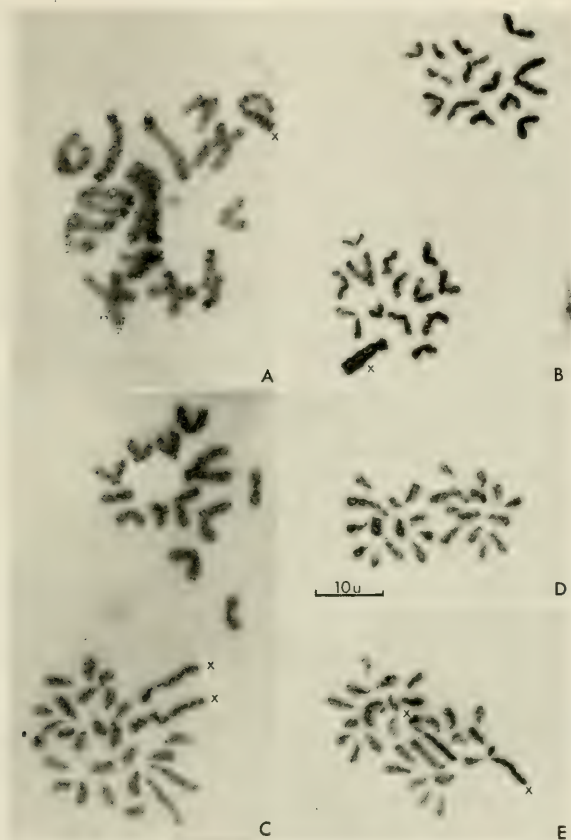


FIGURE 2. Chromosomes of *Coptopteryx viridis*. A) Diakinesis. B) two metaphases of the second meiotic division (with 13 and 13-X chromosomes). C). A metaphase of the second meiotic division with 13 chromosomes and an anaphase with 28 chromosomes. D) Telophase with 13 and 13 chromosomes. E) Telophase with 13 and 13-X chromosomes.

This evidence gave rise to two possibilities: either the existence of an annual-biennial cycle gave rise to two types of eggs, some with a slower development than others, or the development of the eggs after a certain stage had ceased for some reason (the structure of the ootheca would have allowed them to remain fresh). Kume and Dan (1968) observed in *Paratenodera aridifolia* that in the diapause

coincident with hibernation the embryos were in the same stage of development as ours.

It was decided to wait until the next spring to see which of the two alternatives was correct. The first proved to be valid, since on 22-Nov-77 nymphs started hatching from the parthenogenetic oothecas laid during the Summer-Autumn period of 1976 and which had already hatched nymphs in the Spring-Summer of 1976-1977; nevertheless the birth frequency was very low.

The next step was to determine the karyotype of the non-parthenogenetic individuals of this species. It was found that the male has 13 autosomes and a sexual chromosome as haploid number. Figure 1 shows the diploid karyogram of the species. It can be seen that it has acrocentric autosomes of the "t" type according to the classification of Levan, Fredga and Sandberg (1964), and the X chromosomes is subtelocentric of the "st" type according to the same classification.

This karyogram was built from two metaphases of the second meiotic division, with 13 and 13-X chromosomes respectively. As can be seen in the diakinesis of Figure 2a and in many others observed in this study, no trivalent chromosomes were found, which led us to the conclusion that this species belongs to the XX system for the female and XO for the male. Figure 2b shows two metaphases of the second meiotic division, with 13 and 13-X chromosomes respectively.

Figure 2c shows a metaphase of the second meiotic division with 13 chromosomes and an anaphase with 28 chromosomes which is the result of the separation of chromatids from the metaphase of the previous figure (2b). Figure 2d and 2e show two telophases, one with 13 and 13 chromosomes and the other with 13 and 13-X chromosomes respectively, indicating the end of the meiosis seen in figure 2c. All of these, and other figures studied, made us think that this species belongs to the type XX-XO with a diploid number of 27 chromosomes for the male and 28 for the female.

DISCUSSION

The only species studied with this type of reproduction has been *Brunneria borealis*, from Central Texas and North Carolina, which reproduces exclusively by parthenogenesis (White, 1948) and *Miomantis savignii* (Adair, 1925) from Egypt, which reproduces both by parthenogenesis and after fertilization.

It has been seen in *Coptopteryx viridis*, that the beginning of oviposition varies within a very wide range and is related to the moment in which the animals attain the adult stage. This laying would be related to a certain period of the year more than to the age of the individual. This might explain why the female that reached the adult stage at the beginning of summer delayed 80 days in laying her first ootheca, whereas the one attaining maturity at the end of the same season took 30 days in doing so (Table I). From the analysis of the ovaries studied it is not clear whether or not the growth of the oocytes is synchronous with the process of nuclear maturation (meiosis).

The study of the oogenesis of this species will help to clarify this point. It was observed that the chromosome morphology of *Coptopteryx viridis* differs from that of other species of mantids described by White (1941) and Hughes-

Schrader (1943). These authors studied species from Europe, Africa and Central and North America and found that in most of them the autosomes are metacentric or "M" type, according to the classification of Levan (1964), with some of the submetacentric or "sm" type, according to the same classification. Nevertheless, the sexual chromosomes have the same configuration. This shows a marked difference with the chromosomes of *Coptopteryx viridis* where the autosomes are acrocentric or of the "t" type and the X chromosome is subtelocentric or of the "st" type according to Levan (1964). These data suggest that this is a diploid parthenogenesis, since haploid parthenogenesis never yields females. From this point, two possibilities arise: that the parthenogenesis is automictic or apomictic. In the first case the parthenogenesis would be exclusively thelytoky because of the sexual configuration of the species. In the second case, and due to the low number of hatched nymphs, it could be a thelytoky or amphitoky parthenogenesis. This last type of parthenogenesis would yield males by abnormal meiosis, as occurs with the Aphides (Homoptera) which have a XX-XO sexual system (Lees, 1961). This did not occur in *Coptopteryx viridis*, therefore we are inclined to think that this is a case of diploid parthenogenesis, automictic or apomictic, but exclusively thelytoky. Nevertheless, due to the low viability of the individuals, it is possibly an automictic parthenogenesis, since homocytogosis favors the expression of deleterious genes.

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SUMMARY

Several years of observations of the behavior of the mantid *Coptopteryx viridis* suggested evidence of parthenogenesis in this species. *C. viridis* is a solitary, sedentary animal, where the female often kills the male before copulation takes place, and the average male adult life is half that of the female.

Virgin females were reared in our laboratory from their hatching to the end of their lives; these laid oothecas. From these oothecas, parthenogenetic nymphs were born, all of the female sex and with a very low viability. The karyotype of the non-parthenogenetic individuals of this species was found to be XO-XX with a diploid number of 27 chromosomes for the male and 28 for the female. The autosomes were acrocentric or "t" type while the X chromosome was subtelocentric or "st" type, according to Levan's classification.

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DEVELOPMENTAL PATTERN AND ADAPTATIONS FOR REPRODUCTION IN *NUCELLA CRASSILABRUM* AND OTHER MURICACEAN GASTROPODS¹

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An extensive literature about development in muricacean gastropods from the northern hemisphere and tropical coasts has been developed; however, many of these works are strongly descriptive and do not always present an adaptive interpretation of features observed. In recent years a valuable advance has been achieved by Spight, based on his extensive field experience with thaidids. Some muricid developmental rules proposed by Spight refer to factors influencing pre-hatching time, ecology of hatching size, hatching type in relation to latitude and habitat conditions as well as factors conditioning selection of spawning sites (Spight, 1975, 1976b, 1977a, c).

To determine how far these rules are applicable in controlling the evolution of developmental patterns in muricaceans requires more extensive comparative knowledge of species from geographical areas not yet explored. In this sense, the coast line of Chile is very promising, as it comprises an extensive latitudinal range accompanied by great diversity in habitat conditions. Some years ago, a comparative study of reproduction in Chilean muricids was begun. Preliminary interest was focused on the commercially important species *Concholepas concholepas* (Gallardo, 1973; Gallardo, in press). Later, egg masses, embryo feeding and hatching type of *Chorus giganteus* were also analyzed; these results have been discussed in relation to habitat conditions (Gallardo, in press). In the present paper, studies on egg masses and embryos of the intertidal snail *Nucella crassilabrum* from the locality of Mehuín, a small bay near Valdivia, are reported; this information is complemented with field observations on habitat, spawning sites, embryo mortality and pre-hatching time at two different seasons. A discussion follows in order to interpret some of these features; hatching type and hatching size are analyzed in relation to the rules earlier set out by Spight. Emphasis is given to embryo feeding patterns and their possible adaptive significance within the holobenthic muricaceans, a question still not completely answered.

MATERIALS AND METHODS

Egg capsules of *Nucella crassilabrum* were collected on the intertidal rocky shore of Mehuín (39° 25' S, 73° 10' W) from March, 1976 until April, 1977. Capsules of one cluster were separated and monitored to record capsule size composition as well as synchronism of development between different capsules in each group. Development of embryos was followed by opening capsules in different

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FIGURE 1. *N. crassilabrum*. Egg masses attached on vertical rocky substrate at the intertidal of Mehuín.

embryonic phases. Measurements and drawings were made from living material observed under a stereo-microscope provided with a micrometer eyepiece. Number of nurse eggs ingested per embryo was estimated from capsules in which all nurse eggs had been eaten; careful dissections of embryos in early trochophore phase made it possible to determine the number ingested and its variation.

Pre-hatching time was recorded in clusters that were spawned at different seasons of the year. For this purpose, two egg masses containing eggs in early cleavage stage were selected and tagged. One of these was spawned during late autumn and the other during late spring. Each spawning site was periodically visited on tidal exposure periods until the snails had hatched; on each visit, 3 or 4 capsules were collected for further examination at the laboratory. Water temperature of the sea is recorded daily at the Laboratory of Mehuín; this is the information used for the present paper. Spawning sites used by *N. crassilabrum* were inspected to record habitat preferences. Embryos killed by physical stresses were identified by their pink color.

RESULTS

Egg capsules and masses

Shape of the egg capsule of *Nucella crassilabrum* resembles that of its congener, *N. lapillus* (Ankel, 1937). Each flattened capsule has concave and convex sides; when seen from either the convex or concave side, the structure above the peduncle appears nearly oval in shape, with a gradual increase in breadth toward the top. At the top of the capsule there is a circular exit hole which is closed by a

prominent plug. It is possible that, as observed in other neogastropods (Hyman, 1967), this plug weakens and dissolves as the embryos reach the hatching stage. The exit hole diameter is approximately 875 to 1200 μm . Inside the capsule, eggs and mucus-like fluid are contained in a thin transparent sac. Each capsule possesses a short stalk; stalks of various capsules are cemented to the substrate in a continuous band. The capsule wall is fairly transparent, showing the embryos inside. Clusters are yellowish, due to the yellowish eggs when freshly laid, and fade to dull grey with the development of the larvae. Size of egg capsules depends on size of the female producing them. The length of the capsules we have observed, excluding the stalk, varies between 5.0 to 12.8 mm.

The capsules are laid very close to each other with a distance of approximately 2.0 to 2.5 mm between stalks. Field observations suggest that, as typical in certain muricids, communal spawning is also the rule in this species. The clusters (Fig. 1) are laid close to each other, making it difficult to ascertain the number of egg capsules laid by a single female. Nevertheless, in certain cases, the orientation and capsule size allow one to distinguish egg masses laid by different females; such clusters may contain up to 60 egg capsules. Capsules of the same cluster are arranged in a definite pattern, all of them facing the same direction; capsules arranged in a given row alternate in position with respect to those from a contiguous row as seen in Figure 1. Direction in which the different clusters are oriented on the substrate appears random.

Spawning sites

Egg masses are attached to rocky substrates, most frequently in crevices and on vertical surfaces and least frequently in tidepools and on horizontal surfaces. In general, permanently wet and shaded intertidal sites, from extreme low water of spring tides to about mean low water of neap tides, are preferred. Capsules at this last intertidal level were found in a group of rocks partially buried in the soft sandy beach. In this area, extreme seasonal sand fluctuations of about 80 or 100 cm are observed and a great quantity of sand is deposited during the summer, greatly reducing the rocky surface merging above the soft bottom. In that case, many egg masses of *Nucella crassilabrum* are covered by the sand. Furthermore, the retreating tide regularly exposes them to dry air and wind, especially throughout late spring and summer; snails become exposed to air longer and more frequently than lower on the shore and many egg masses do not complete development, probably owing to the effect of these environmental stresses. Capsules with embryos killed by physical stresses change from the normal yellow color to pink or purple. The most favorable situation for subsequent hatching of capsules was at those sites where they were permanently submerged in the sea water.

Eggs and embryos

The eggs are creamy white, their diameter varying between 204 and 293 μm with a mean of 240 μm . The number of eggs per capsule varies from 134 to 1116. A significant correlation observed between capsule length and number of eggs per capsule (Fig. 2) accounts for this variation. Besides the normally viable eggs there are many others that undergo atypical development and serve as nurse eggs.

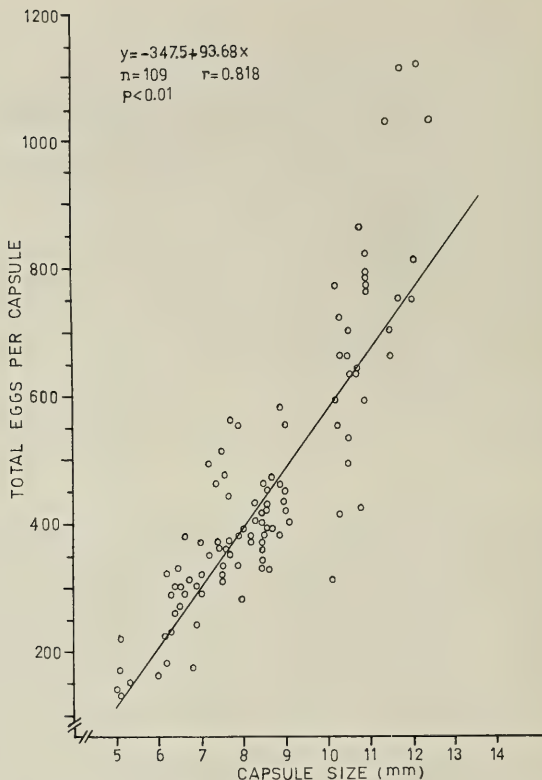


FIGURE 2. *N. crassilabrum*. Relationship between number of eggs per capsule and capsule size.

Counts in three egg capsules of different sizes (Table I) reveal that only 6.6 to 7.9% of the total eggs in a capsule are viable. The number of embryos also increases in proportion to capsule size (Fig. 3); it varies between 10 and 122.

The intracapsular development of *N. crassilabrum* is of direct type. In viable embryos it follows the normal spiral pattern of cleavage. On the other hand, atypical cleavage is observed in non-viable nurse eggs which appear very variable in shape. The atypical development of these nurse eggs is finally arrested. The fertile larvae begin ingesting nurse eggs when they attain an early trochophore stage. By then, the mouth, esophagus and body expand to enclose entire nurse eggs as they are pushed down the digestive tract by the cilia lining it. The

TABLE I.

Nucella crassilabrum. Percentage of viable embryos and nurse-eggs in three different capsules.

Capsule size (mm)	Total nurse eggs	Viable embryos and percentage
7.9	406	32 (7.3%)
6.8	342	24 (6.6%)
8.6	397	34 (7.9%)

ingested eggs are visible as distinct bulges in the body wall (Fig. 4b) and will spill out intact from an embryo opened during the feeding period. In the succeeding stages (Figs. 4c, d) and intracapsular veliger is gradually developed; by then, the embryos show the appearance of the shell and foot and the velar lobes are partially expanded. The embryo is now clearly divided into head (anterior to the shell), foot (ventrally), and visceral hump (covered by the shell); nurse eggs ingested still obscure details of internal structure (Fig. 4e). The posterior

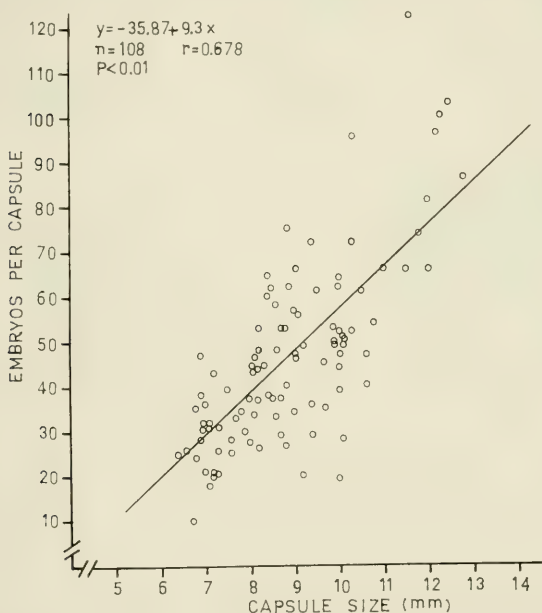


FIGURE 3. *N. crassilabrum*. Relationship between number of viable embryos per capsule and capsule size.

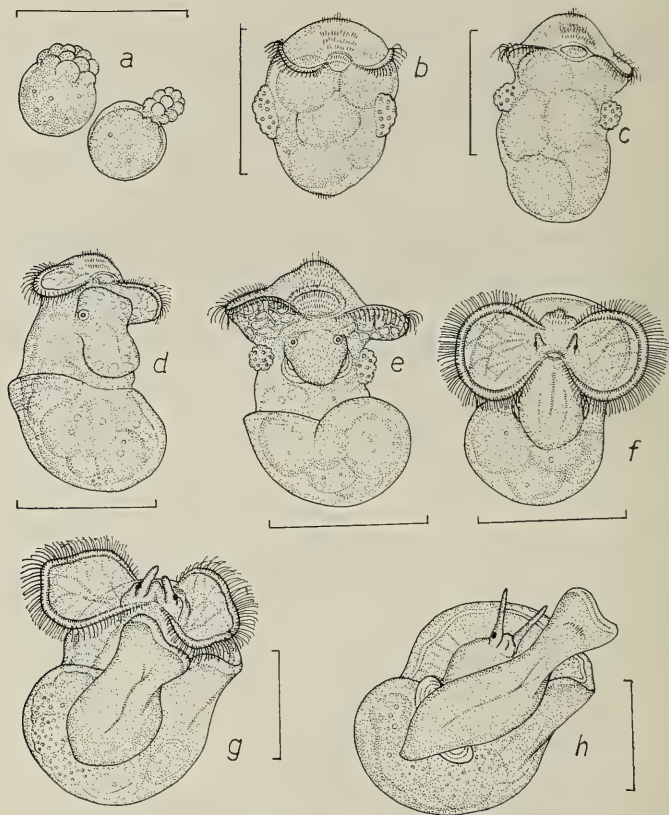


FIGURE 4. *N. crassilabrum*. Different stages of intracapsular development. a) a fertile egg (left side) next to a nurse-egg, in cleavage stage. b) trochophore stage with the outlines of whole nurse-eggs seen through the body wall. c-d) early veliger stage. e-f) mean and advanced veliger stage. g) pre-hatching stage with the velum in reabsorption process. h) hatching juvenile stage. The lines equal 500 μ m.

face of the foot gradually differentiates a small, thin operculum. On either side of the foot a spherical statocyst is visible. Within the velar rim and around the stomodaeum, rudiments of the adult head are now beginning to become organized and a pair of black eyespots is visible on the base of recently developed tentacles

(Fig. 4f). Ingestion and feeding activity may continue in some capsules up to the veliger stage illustrated in Figure 4e.

The next stages of the intracapsular veliger (Figs. 4g, h) show a gradual and extensive increase in size, especially of the shell, foot and tentacles. The yolk gradually disappears in the visceral hump with accompanying differentiation of the viscera. A columella muscle faintly visible on the left side of the visceral hump is able to effect withdrawal of both head and foot into the shell. By the end of intracapsular development, the lateral lobes of the velum have been resorbed and this pre-hatching juvenile shows an active crawling foot and large tentacles.

Embryonic feeding and rate of development

During intracapsular development, some embryos acquire more nurse eggs than their capsulmates and this is reflected in the size distribution of embryos and hatchlings. Embryos in trochophore phase as small as $450\ \mu\text{m}$ were found together with others as large as $775\ \mu\text{m}$ after all nurse eggs had been eaten. By this time, the number of eggs eaten by each larva varies from 3 to 20 as shown in Figure 5 (left side) for three capsules with different numbers of capsulmates. This figure also shows the frequency at which embryos ingest different numbers

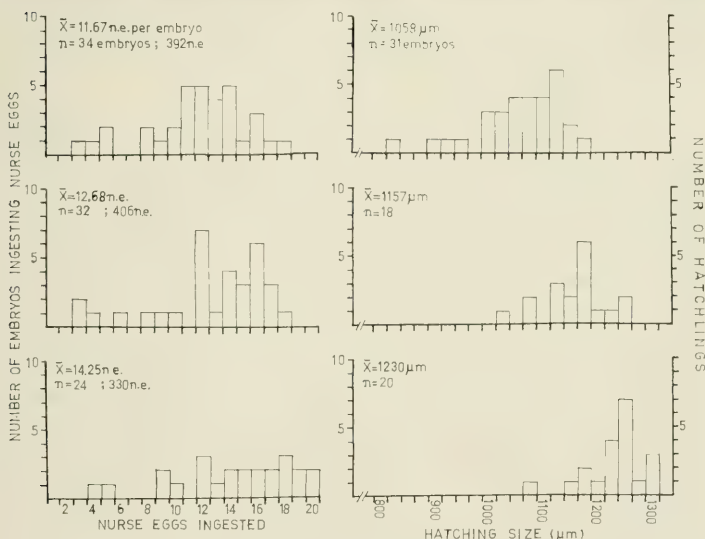


FIGURE 5. *N. crassilabrum*. Left, frequencies of embryos ingesting different numbers of nurse-eggs in three different capsules. Right, hatching size frequencies between embryos of three different capsules. n.e. = nurse-eggs.

of nurse eggs. Hatching size distributions for juveniles from three different capsules are shown in Figure 5 (right side). While some juveniles attain 825- μ m shell length at the moment of hatching, others are as large as 1300 μ m. Mean number of nurse eggs ingested per embryo per capsule as well as mean hatching size, seem to change regularly as the number of embryos per capsule decreases. The effect of haphazard distribution of embryos among capsules on hatching size, has been previously studied by Spight (1976a) in the muricids *Thais emarginata* and *Acanthina spirata*. While embryo counts are less predictable in these species, nurse eggs per capsule are distributed more regularly. Some embryos share their yolk supplies with many more capsulmates than others and this factor is most important in determining a variable hatching size between nurse egg feeders' embryos (Spight, 1976a).

Time required to complete intracapsular development was estimated from two egg masses spawned at different seasons of the year. At the time the observations began, the egg capsules were in an early cleavage stage. An egg mass controlled during autumn and winter months took between 70 and 80 days to fully develop and hatch; mean of monthly water temperature from May to August, 1976 at Mehuín, varied between 9.67 and 10.60° C. In other masses that developed during late spring and early summer, the time was significantly reduced to approximately 55 to 65 days; in this case, mean of monthly water temperature from November, 1976 to January, 1977 varied between 11.40° and 14.10° C. Doubtlessly, differences in developmental rates in nature are well accounted for by seasonal changes in sea water temperatures at Mehuín.

DISCUSSION

Nucella crassilabrum shows the most evolved pattern of development known for Chilean muricids if we compare it with observations in *C. concholepas* (Gallardo, 1973) and in *Ch. giganteus* (Gallardo, in press). The type of development of *N. crassilabrum* agrees with that reported for its congener *N. lapillus* (Pelseneer, 1910). Such pattern of development is expected in high latitude muricaceans inhabiting intertidal rocky shores (Spight, 1977a). Spight establishes that, among the rocky intertidal muricaceans, the hatching type evolves markedly according to a latitudinal gradient; while in this habitat all tropical species maintain planktonic larvae, all high latitude species metamorphose before hatching. In this sense, *N. crassilabrum*, whose geographic distribution extends along the Chilean coast to the Magellan Strait, may be included within the muricid group showing such reproductive tendency. In a paper about hatching type of *Chorus giganteus* (Gallardo, in press), a case of coincidence with Spight's predictions for muricaceans from another type of habitat has also been reported. According to Spight (1977a), muricaceans with an intermediate hatching type (a non-feeding veliger near to metamorphosis) had been found to inhabit a shallow water sand habitat; the findings in *Ch. giganteus* agree, as this sand bottom muricid also possesses such hatching type. Causes for these tendencies in muricid developmental patterns when related to latitude and habitat conditions are unknown.

All reproductive patterns should involve adaptations to maximize survival of embryos and, consequently, their reproductive fitness. Some of these adaptations

should include ability of adults to discriminate and choose good spawning sites, as well as an appropriate developmental time and hatching size. Our field observations in *N. crassilabrum* reveal that mortality of embryos is of common occurrence at least at the upper shore spawning sites. Probably some sites offer more protection than others; for instance, one would expect physical stresses to be minimal in tidepools, but they are used less frequently than vertical surfaces exposed to repeated dehydration during low tides. According to observations of the intertidal muricid *Thais lamellosa* (Spight, 1977c), spawning sites selected would reflect the conflicting demands of different life history stages. According to Spight, vertical surfaces are least accessible to predators and often are covered with food (barnacles), but provide little protection from physical stresses. Crevices offer some protection from physical stresses and usually are covered with barnacles but are accessible to predators. Understone surfaces and tidepools offer protection from stresses but are accessible to predators and are often distant from food sources. With these considerations in mind, Spight concludes that *T. lamellosa* females deposit capsules most frequently on the surfaces that are most suitable when the demands of all life history stages are considered. Studying the congeneric species *N. lapillus*, Feare (1970) found that physical stresses operating during exposure to air are responsible for hatching success of 0% at mean tide level and 57% at mean low water of neap tides. Our field observations in *N. crassilabrum* suggest that this species also uses poor spawning sites regularly and it probably does so for the same reasons that *T. lamellosa* does. For instance, different environmental stresses seem to be influencing survival of *N. crassilabrum* adults when closely located sites are compared (Gallardo, in preparation); demands of this life history stage could be in part conditioning the selection of spawning sites that are most suitable.

Hatching size of intertidal muriceans is shorter (0.6 to 1.3 mm; $\bar{X} = 1.01$ mm) than that of species living in subtidal habitats (0.6 to 2.5 mm; $\bar{X} = 1.54$ mm) or on coral reef flats (1.1 to 1.9 mm; $\bar{X} = 1.40$ mm) [Spight, 1976b; ($\bar{X}$) = mean values calculated from a few species where data are available]. According to this, the hatching size of *N. crassilabrum* (0.82 to 1.30 mm) corresponds with the habitat this muricid occupies. To attain the appropriate hatching size, holobenthic muriceans have followed two evolutionary paths: provision of nurse eggs or increase in size of a self-sufficient fertile egg. Now the question arises: which selective forces favor one or the other of these developmental options? It has been hypothesized that providing much of the yolk as nurse eggs may allow a snail to have a large hatching size and at the same time a relatively brief developmental time (Spight, 1975). In order to prove the validity of this hypothesis between muriceans, we may use information available about developmental time, hatching size and embryonic feeding source both in *N. crassilabrum* (this paper) as in other species (Spight, 1975, 1976b); this information is compared in relation to habitat. We may see that among intertidal muriceans adaptation for nurse egg feeding is of common occurrence; in a few species of this habitat, energy for developing embryos is totally obtained from larger fertile eggs (*Thais lamellosa* 590 μ m; *T. canaliculata*, 620 μ m). *T. lamellosa* reveals a developmental time of 67 to 91 days at 9.6° to 11° C, more or less similar to that of *N. crassilabrum*, although the upper limit of its range, 91 days, slightly exceeds that of *N. cras-*

silabrum; 80 days at 9.6° to 10.6° C. On the other hand, subtidal species usually require a large hatching size. To attain it, they usually have evolved a large fertile egg (*Ceratosoma foliatum*, 720 μm ; *Torvanurex territus*, 675 μm); numerous nurse eggs per embryo are consumed in species of this habitat showing such feeding mechanism (*Murex senegalensis*, 35 n.e. per embryo; *M. quadrifrons*, 91 n.e. per embryo). Subtidal species whose egg exceeds 600 μm in diameter, reveal a markedly slow developmental rate; for instance, *T. territus* takes 90 days to hatch at 20° C and *C. foliatum* takes 120 days to hatch at 10° to 12° C. Our observations in *N. crassilabrum* and those of Spight (1977c) and Feare (1970) reveal that one of the most important sources of embryo mortality in muricaceans laying in the intertidal, are the physical stresses operating at this level; an embryo of *T. lamellosa* cannot tolerate even one two-hour exposure (Spight, 1977a). Similar observations have been made by Pechenik (1978) in prosobranchs of the family Nassidae. Pechenik found that egg capsules of *Nassarius obsoletus* did not afford substantial protection against desiccation; 0.5-hr exposures to 75% relative humidity killed 17.5% of the embryos. It is reasonable to assume that such mortality would be reduced by laying the egg capsules in protected micro-environments, as *N. obsoletus* clearly does (Pechenik, 1978), or by shortening developmental time of clusters spawned at more exposed sites of the intertidal. When, for reasons considered above, an intertidal muricid does not always use good spawning sites for embryos, the possibility that it is shortening developmental time should be preferred. An evolutionary option in this sense may be to change to another form of embryonic feeding, that is the provision of nurse-eggs. But, how to explain nurse-egg feeding by some subtidal muricaceans? In this case, this embryonic feeding adaptation seems to have been specially favored to attain an extremely large hatching size. Relatively large muricid eggs range from 675 to 920 μm in diameter (*T. territus*, *Thais lima*); nevertheless the hatchlings developed from such eggs do not exceed from 1.3 to 1.5 mm in length. In subtidal species, usually requiring a larger hatchling (1.6 to 2.0 mm), each embryo is provided with numerous nurse eggs (*M. quadrifrons*, *M. senegalensis*). These preliminary considerations suggest that a delicate compromise between at least the optimal hatching size and developmental time, could be orienting evolution of embryonic feeding adaptations among holobenthic muricaceans. However, further work is necessary to reinforce this hypothesis. The results for the small number of species considered in this discussion cannot, by themselves, support it, but they can add to the data for future comparative studies. Information about developmental time, hatching size, habitat, and mortality sources is still lacking in various muricids. An optimal material to evaluate the advantages of evolving nurse eggs is offered by *T. canaliculata*; this intertidal snail appears to be in the course of evolving from one reproductive mode (self-sufficient large egg) to the provision of nurse-eggs for embryos (Spight, 1977b).

SUMMARY

1. Eggs of *Nucella crassilabrum* range from 204 to 293 μm in diameter (mean = 240 μm). Only 6.6 to 7.9% are fertile; the remaining are ingested as nurse eggs.
2. Embryos metamorphose before hatching. Pre-hatching time ranges from 55 to 80 days according to seasonal temperature fluctuations.

3. Hatching size varies from 0.82 to 1.3 mm, depending on number of nurse-eggs ingested per embryo (from 3 to 20). Number of fertile embryos per capsule (10 to 122) depends on capsule size.

4. Hatching type and hatching size shown by *N. crassilabrum* agree with those of other muricaceans living in similar habitat conditions.

5. Pre-hatching time and hatching size data of various muricaceans are analyzed to determine to what extent they influence embryonic mode of nutrition, namely the presence of nurse-eggs or alternatively large and fertile self-sufficient eggs. Provision of nurse-eggs for embryos is of common occurrence among intertidal muricaceans and this mode of nutrition seems to have been favored in such habitats to reduce developmental time. Providing the yolk as nurse-eggs seems also to contribute to a larger hatching size, as suggested by some subtidal muricaceans with such embryo support patterns.

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EARLY POST-METAMORPHIC GROWTH, BUDDING AND SPICULE FORMATION IN THE COMPOUND ASCIDIAN *CYSTODYTES LOBATUS*

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Compound ascidian colonies are comprised of many individuals wholly embedded in a common tunic. Budding is accomplished by active epidermal synthesis and constriction, with the regenerating buds moving through this common tunic to form a new system (see Berrill, 1951, 1961 for reviews of early papers; Sebastian, 1957; Levine, 1960; Freeman, 1971; Nakauchi, 1966a, b, c, 1970, 1977; Nakauchi and Kawamura, 1974a, b, 1978). *Cystodytes*, unlike other genera in the suborder Aplousobranchia, family Polycitoridae, is distinguished by large numbers of calcareous spicules surrounding the abdomen of each zooid, forming a spicular sac separating each individual from neighboring zooids (Ritter, 1900; Van Name, 1945). During budding this spicular sac must be reorganized and reformed around the abdomen of each bud, but the way in which this is accomplished is completely unknown. The common tunic overlying the spicular layer contains numerous large, closely packed, acid-filled bladder cells (Abbott and Newberry, 1980); any rupture of these cells results in immediate dissolution of nearby spicules and evolution of CO₂. The spicules might be dissolved and reformed during budding or just reallocated in some way among the buds; there appear to be no life history studies on any species of the genus *Cystodytes*, even though the genus is common and widespread (Van Name, 1945; Millar, 1975). Therefore, the present study is an examination of the general ecology and method of budding in *Cystodytes lobatus* (Ritter, 1900), a species that occurs abundantly in the low intertidal and subtidal zones along the central California coast. The time course of tadpole release, spicule formation in oozoids (the individuals developing from newly-settled tadpoles before the first budding), and the method of budding with each zooid isolated in its own spicular sac are discussed here. Included is the discovery that the spicules are contained within a discrete extra-cellular membrane. This membrane has been named the tunic spicular lamina and is concluded to form an organic matrix for spicule formation.

MATERIALS AND METHODS

This work was carried out at the Hopkins Marine Station, Pacific Grove, California, between February and August, 1978. Animals were collected from rocks in front of the marine station and at Pt. Pinos, about two miles away. Three color variants exist: white, pink and orange. Since the taxonomy of these variants has not been studied, and since Ritter (1900) based his original description of the species on the white form, only white colonies were used throughout this study.

Colonies were maintained in unfiltered running sea-water aquaria in the laboratory; between February and August the temperature varied only from 13° to 16° C. Colonies usually lived at least a month in the laboratory, and if they reattached to the bottom of the aquarium they survived even longer.

Colonies with tadpoles were easily recognized by the presence of the bright pink yolky embryos. Since it is known that some colonial ascidians release their tadpoles in response to light (*e.g.*, Watanabe and Lambert, 1973), the colonies were kept in the dark at night. Tadpole collection was accomplished by removing the colonies in the morning to a clear plastic aquarium supplied with running sea water. Drainage was through a hose penetrating the aquarium at one end, with water flow adjusted so that the water level was at the hose exit. In this way, as tadpoles were released from the colonies and swam upwards they were skimmed off and carried through the hose to a tadpole collector, consisting of a short piece of polyvinyl chloride pipe with 300- μ m Nytex glued to the bottom of it and resting in a small dish. Tadpoles were released throughout the day. Periodically they were removed from the collector to a petri dish with a glass slide in the bottom of it centered over a piece of black plastic under the dish to induce settling of the tadpoles in the darkest area. These slides were then placed in a plexiglass slide holder and submerged in a large cement tank in the laboratory filled with running unfiltered sea water where they were maintained for several months. Slides were removed from the holder and placed in a sea water-filled petri dish for examination of the living zooids, using an American Optical dissecting microscope with phototube. All photographs were taken with an Olympus OM-2 35-mm SLR camera with microscope adapter.

A Beckman Expandomatic IV pH meter equipped with a rapid-response MI-410 combination pH probe (Microelectrodes, Inc., Londonderry, N. H.) was used for all pH determinations. Bladder cell pH was determined in two ways. Adult colonies were washed several times in distilled water, blotted dry with Kimwipes, then either the pH electrode was carefully inserted about 2 to 4 mm into the superficial layer of the tunic or bladder cells in the upper tunic layer were broken by agitation with a fine probe and then the pH electrode was immersed directly into the resulting pool of (mostly) bladder cell fluid. Results were the same for both methods. Bladder cell contents were analyzed for the presence of chloride and sulfate ions by probing them directly with hand-made finely drawn out glass micropipettes under a dissecting microscope. The fluid collected was expelled into a watch glass, and a drop of either silver nitrate or barium chloride was added. (AgNO_3 forms a precipitate of AgCl_2 in the presence of chloride ions, BaCl_2 forms a precipitate of BaSO_4 in the presence of sulfate ions.)

Because alcohol dehydration and even fixation in buffered formalin did not prevent rupture of bladder cells and dissolution of spicules, razor blade sections of living colonies were made and stained supravitaly with PAS, aldehyde fuchsin, alcian blue pH 2.5, alcian blue pH 1.0, sudan black B, 0.1% toluidine blue, or neutral red, using the methods in Pearse (1968), in order to analyze the tunic for acid mucopolysaccharides and other structural materials.

A few 4-wk-old oozoids settled on slides were relaxed in 100 ml sea water to which had been added 1 drop of menthol-saturated 95% ethanol, according to the method of Abdel-Malek (1951). When total relaxation was achieved in 3 to

6 hr, 10% formalin was added drop by drop while stirring, until the animals were dead. The zooids were transferred to 10% formalin for 24 hr, then removed from their slides, washed, dehydrated in alcohols and embedded in the Polysciences JB+ plastic embedding medium. After 24 hr the plastic blocks were trimmed and sectioned at 1 to 2 μm . Sections were removed one at a time, placed in a drop of 1% ammonia on a slide to flatten out, then flame-dried and stained with methylene blue or 0.1% toluidine blue.

RESULTS

General biology

Cystodytes lobatus grows in large mats, up to a half meter across or more; whether each mat is one colony or many that have fused or abutted was not determined. Colonies by the Hopkins Marine Station averaged 5 mm in thickness. However, material collected elsewhere and in D. P. Abbott's private collection may be up to 1 cm or more in thickness (Abbott and Newberry, 1980).

Colonies always occurred in the low intertidal or subtidal; a -0.6 ft tide or lower was necessary to collect intertidally. The best intertidal sites were under overhanging rocks and to a lesser extent on the north side of vertical rocks, away from direct sunlight and (possibly) from competition with plants.

Large colonies were commonly observed to have overgrown many barnacles and polychaete worm tubes, resulting in a mat with many superficial ridges and knobs. Cross sections indicated colonies to be of variable thickness; some ridges covered overgrown barnacles while others were present without any underlying irregularity of the substratum. Other compound ascidians, common in the same area as *Cystodytes*, were *Aplidium californicum* and *Archidistoma psammion*; colonies of both these species also attained large size, and a dynamic situation appeared to be present of overgrowth of one ascidian by another—either overgrowth of *Cystodytes* by *Aplidium* or *Archidistoma* or vice versa.

Very few predators were observed feeding on *C. lobatus*; those animals found on or near colonies in the field were the starfish *Patiria miniata* and the gastropods *Calliostoma ligatum*, *Tegula funebris*, *Megathura crenulata*, and *Lamellaria diegoensis*. However, these observations were made intertidally at low tide when *Cystodytes* was out of water; high tide observations might be different. When *Cystodytes* is out of water it forms a slimy coating of an (apparently) mucus-like material that can be seen hanging in long strands and dripping off the colonies. This material is not present in submerged colonies until the surface is rubbed. The significance of this is as yet unknown.

Laboratory feeding observations in the above-mentioned animals found on or near colonies showed that all but *Tegula funebris* would eat *Cystodytes* in the laboratory. Stomach contents of subtidal *Calliostoma ligatum* and *Megathura crenulata* collected on or near *Cystodytes* yielded chunks of colony with the spicules mostly intact (Sellers, 1977 and personal communication). *Lamellaria diegoensis* was maintained in the laboratory from March 10 until August 31 on a diet of nothing but *Cystodytes lobatus*; during this period its weight increased from 2.2 grams (May 3) to 6.0 grams (August 21). A study of feeding of

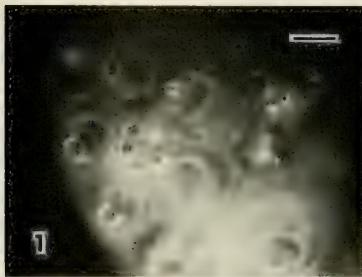


FIGURE 1. Two *Cystodytes lobatus* systems comprised of 5 and 3 zooids. Scale bar 1 mm.

Lamellaria on *Cystodytes*, including a calorific analysis of *Cystodytes* colonies utilizing a semimicro bomb calorimeter, will be published separately (Lambert, 1979).

Other animals associated with *Cystodytes* as well as other colonial ascidians are the clam *Mytilimeria nuttalli* and the amphipod *Polycheria osborni*, both of which live embedded in the ascidian test (Abbott and Newberry, 1980). Very little is known of the relationship of these species to their ascidian hosts (Skogsberg and Vansell, 1928; Yonge, 1952).

Colony organization and tadpole release

The zooids of *Cystodytes* (Fig. 1) are arranged in systems (Van Name, 1945) with a mean of 4 or 5 zooids per system (Fig. 2). The atrial siphons open separately at the surface (Fig. 1). The abdomens are surrounded by a layer of overlapping calcareous spicules (Figs. 3, 4) and the test matrix is filled with bladder cells (Van Name, 1945) tightly packed and ranging in size roughly from 35 to 80 μm . These cells are filled with sulfuric and possibly hydrochloric acids; tests were positive for the presence of both sulfate (Abbott and Newberry, 1980)

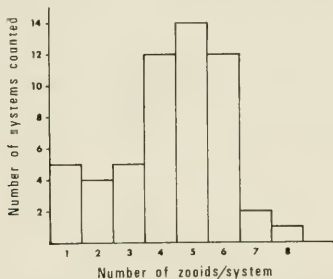


FIGURE 2. Number of zooids per system. $n = 55$ systems, $\bar{x} = 4.36$ zooids/system, $s.d. = 1.69$.

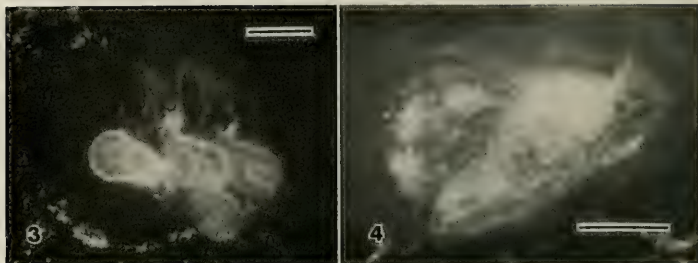


FIGURE 3. Underside of a *C. lobatus* system. Scale bar 1 mm.

FIGURE 4. *C. lobatus* oozoid 32 days old. Scale bar 0.5 mm.

and chloride ions. The pH of bladder cell contents was determined to be 1.3.

Embryos develop within the atrial chamber of the adult zooids (Van Name, 1945). Because of their bright pink color they could be observed easily and were recorded in nearly all colonies collected between March 7 and August 18.

Tadpoles were collected in the tadpole collector and settled on glass in order to observe the growth of zooids in the laboratory, the time course of spicule and bladder cell formation, and the method of budding. The time course of tadpole release (Fig. 5) reveals that more tadpoles were released after 3 to 4 hr in the light following overnight dark adaption under black plastic than at any other time, and tadpoles were released more or less continuously all day. Colonies left in an uncovered aquarium overnight in order to observe their reaction to natural dawn also released larvae sporadically throughout the day.

Cystodytes tadpoles are large, with a mean body length of 1.27 mm ($n = 25$, s.d. = 0.113), tail length of 2.59 mm ($n = 25$, s.d. = 0.141), and tail width of

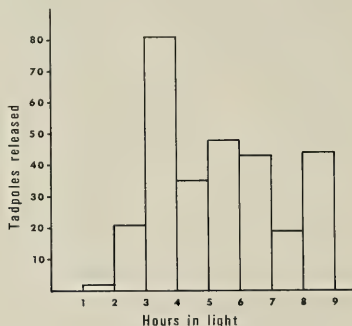


FIGURE 5. Tadpole release as related to duration of light period after darkness. Numbers are totals for 10 days of observations.

0.76 mm ($n = 25$, s.d. = 0.049). The heart beats somewhat erratically but does reverse, as in the tadpoles of *Distaplia* and *Diplosoma* (Cloney, personal communication) and *Pycnoclavella stanleyi* (Trason, 1963). Bladder cells are already densely packed in the test matrix of the tadpole. The pH of six individuals homogenized in a few drops of distilled water was 2.85. A few tadpoles metamorphosed within 15 min of being released, though most had a free larval life of 1 to 3 hr. Settlement was greatly enhanced by using slides that had been soaked in sea water for several days.

Post-metamorphic growth and budding

Young oozoids begin to feed 3 to 4 days after settlement (as determined by the presence of food pellets in the gut) and by one week of age the gut has differentiated into five well-defined regions similar to those in many aplousobranchs: esophagus, stomach, post-stomach, mid-intestine, and intestine or rectum. The stomach and intestine are orange-brown; the rest of the zooid is colorless or nearly so. By 24 hr or so after settlement, oozoids have four rows of stigmata, the same as the adult blastozooids. This is similar to the closely related polycitorid *Archidistoma ritteri* (Levine, 1960) in which both oozoids and blastozooids have three rows. In the polyclinid *Amaroucium multiplicatum* (Nakauchi, 1966a), in contrast, the oozoids have four rows but after budding the blastozooids have six or seven rows of stigmata. In *C. lobatus* there are usually four stigmata per side in the anteriormost row at metamorphosis; at budding this row has 17 or 18 stigmata per side. With successive buddings the blastozooids orient vertically with the abdomen directly beneath the thorax rather than curved around it is in the oozoid, but otherwise the oozoids and blastozooids appear to be the same morphologically. Whether oozoids ever from gonads, however, was not determined.

There is great variation among oozoids in the quantity of spicules produced, even those arising from tadpoles from a single colony reared on the same slide (compare Fig. 4 with Figs. 6 and 8). A number of tadpoles were collected on May 25 and allowed to settle on three slides which were maintained in the

TABLE I.

Relationship between spicule density and time to budding, number of abdominal buds and time until buds begin feeding in C. lobatus.

	n	$\bar{x}$	s.d.
Days from settlement to budding for zooids with few spicules	32	29.72	3.0
Days to budding for zooids with many spicules	18	37.33	6.28
Number of buds, few-spiculed zooids	32	3.59	0.61
Number of buds, many-spiculed zooids	18	2.89	0.47
Days from budding until buds begin feeding in few-spiculed zooids	24	5.58	0.50
Days from budding until buds begin feeding, many-spiculed zooids	15	5.67	0.62

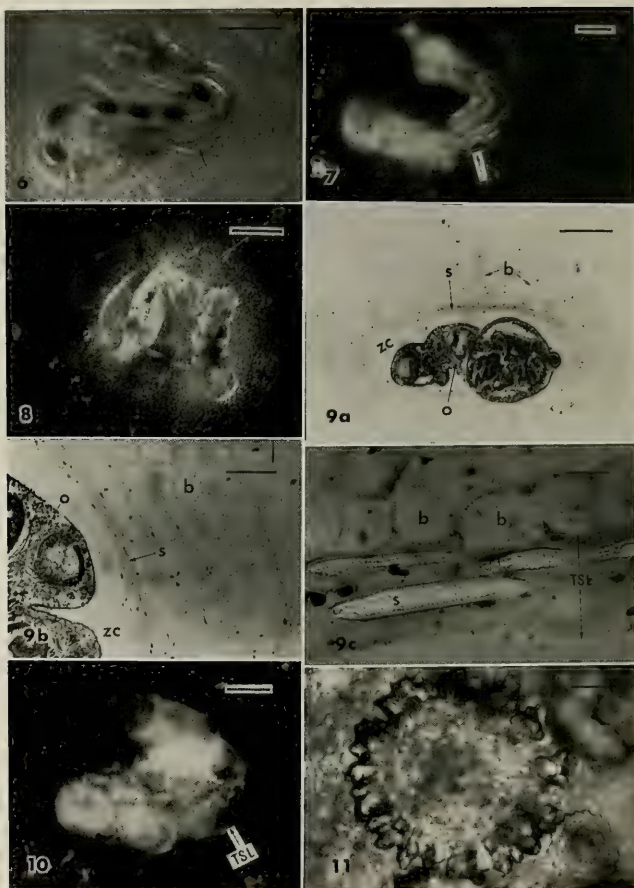


FIGURE 6. Twenty-five-day-old oozoid. Arrow indicates position of new stomach. Scale bar 0.5 mm.

FIGURE 7. Thirty-one-day-old oozoid. Arrow indicates new stomach at posterior end of mother bud. Scale bar 0.5 mm.

FIGURE 8. Thirty-three-day-old oozoid; budding is nearly complete. Scale bar 1 mm.

FIGURE 9. Four-week-old oozoids, 1 μ m sections. (a) entire zooid; scale bar 250 μ m.

(b) Enlargement of tunic region; scale bar 100 μ m. (c) TSL region of tunic; scale bar 20 μ m. b = bladder cell, o = oozoid, s = spicule, TSL = tunic spicular lamina, zc = zooid cavity.

FIGURE 10. Thirty-six-day-old oozoid forming 3 abdominal buds; scale bar 0.5 mm. B = bud, TSL = disrupted tunic spicular lamina.

FIGURE 11. Spicule from an adult *C. lobatus* colony. Scale bar 50 μ m.

laboratory until the end of August. Table I compares oozoids that developed few spicules with those that developed many. Zooids with many spicules took longer to bud (37 days as compared with 30 days) and produced fewer buds. (These are lab times; colonies occurring naturally in the field grew more rapidly than those raised in the lab, and achieved larger size). Without exception, buds always produced the same (subjective) quantity of spicules as the mother had: all buds of a many-spiculed oozoid developed a heavy coating of spicules, and all buds of a few-spiculed oozoid developed only a sparse coating of spicules. This may explain why in some colonies in the field all the zooids have few spicules, while in other colonies all zooids have many spicules.

An attempt was made to study growth rate in a group of zooids by removing slides from the slide holders every other day for measurement of the zooids settled on them. However, this had an obviously detrimental effect on the zooids: the time to budding was longer, only two buds were produced per oozoid, and nearly all the animals ultimately degenerated, so these data were discarded. Other workers contemplating using this technique should be aware of these possible effects.

Although the process of budding has not previously been described for *Cystodytes*, it is in fact similar to budding in other polycitorids (Oka, 1942; Oka and Usui, 1944; Berrill, 1947, 1948; Levine, 1960; Nakauchi, 1966b, c) and in the polyclinids (Nakauchi, 1966a, 1970, 1974, 1977). *Cystodytes* exhibits Nakauchi's (1966a) Type I budding (abdominal; all buds receive some digestive and epicardial tissue). As in *Amaroucium yamazii* (Nakauchi, 1970), budding in *Cystodytes* is preceded by an elongation and enlargement of the posterior end of the esophagus which begins at least 10 days prior to budding (Fig. 6). This esophageal enlargement will become the new stomach of the mother bud (Figs. 7, 8). A few hours before budding begins, the orange-brown stomach and intestine elongate greatly, as does the epicardium. Feeding ceases, the thorax contracts, and all fecal pellets in the digestive tube collect at the end of the rectum. Budding proceeds posteriorly, with the first constriction occurring at the anterior end of the old stomach. Sometimes the heart of this first bud (called the mother or thoracic bud) can be seen beating at this time. Three or four abdominal buds form, with the terminal bud receiving the mother's old heart. All buds receive some of the orange-brown stomach and intestinal tissue as well as some epicardial tissue. After budding is completed and the mother bud's digestive tube has fully regenerated, the thorax relaxes, the old fecal pellets are ejected, and feeding resumes. Buds begin feeding after 5 to 6 days, and the next budding occurs in about 3½ weeks (lab time). Buds usually orient to the mother but one or two might join a neighboring system; this is probably the reason for the large range in number of zooids per system in Figure 2.

Spicule formation and reallocation at budding

As mentioned earlier, a major difference from the Polyclinidae and from other genera in the Polycitoridae is the formation of calcareous spicules in *Cystodytes*. Using a dissecting microscope at 40 or 80× one can distinguish the first spicules as early as 5 days after settlement. At first, the spicules are very tiny and cannot be individually discerned; only a white spot is noticeable constituting the

entire amount of spicular material. Spicule formation always begins on the inner side of the abdomen at the junction between abdomen and thorax. Gradually the spicules appear to migrate as they increase in number and size until at the end of 4 weeks (laboratory growth time) a zooid's abdomen is completely covered with somewhat overlapping non-birefringent spicules (Fig. 4). These spicules are not attached to the abdomen, though; they are embedded in the innermost layer of tunic that lines the zooid cavity. This layer has been named the "tunic spicular lamina" (TSL). Figure 9 shows the newly forming spicules embedded in the middle of the TSL. Above it are the closely packed bladder cells extending all the way to the outer tunic surface, with the common tunic consisting of the material between the bladder cells. The TSL appears to be completely extracellular, because when a razor blade cross section is made through an adult colony, the zooids can be removed easily from the tunic leaving all the spicules behind. The TSL thus forms a spicular sac in the tunic surrounding but separated from the abdomen of each zooid.

The fate of the spicules at the time of budding was next studied, to determine whether they are dissolved and reformed or simply divided up among the buds in some way. A careful examination at 2 to 3 hr intervals of a few budding zooids showed that first the abdominal epidermal constrictions separated the buds within the zooid cavity. When this process was at least partially completed, the spicular sac began to constrict, resulting ultimately in the abdominal portion of each bud being surrounded by a small spicule sac (Fig. 10). At no time was any dissolution of spicules observed; some of the larger spicules could be followed as they migrated to the buds. During the first stages of spicule "reallocation" many of the spicules were disoriented from their previously regular overlapping pattern. Instead of lying parallel to the abdominal epidermis, they might now be perpendicular to it. This would be expected if the spicules were embedded in a membrane and the entire membrane were constricting. A few spicules were sometimes "left behind" in the test, where they remained isolated. However, active and rapid synthesis of new spicules was also occurring during this time, and approximately 3 to 4 days after the onset of budding all buds were close together but separated from one another by their own spicular sacs, with the spicules regularly aligned parallel to the inner edge of the tunic lining the zooid cavity.

New spicules continued to be added as the buds grew, and the old spicules increased in diameter. Indeed, in adult colonies some spicules may be 1 mm or more in diameter; these might have been carried through a number of generations. They exhibit a complex configuration of knobs not found on small, newly formed spicules (Fig. 11).

Histochemistry of the tunic spicular lamina

Hunt (1970) indicated that in molluscs, the formation of calcium carbonate may depend upon induction by an organic matrix with the presence of sulfated acid mucopolysaccharides being important to this process. Recent papers on tunic composition listed these and other substances found in ascidian tunic and the stains appropriate for their detection (Deck, Hay and Revel, 1966; Smith,

TABLE II.
Histochemistry of *C. lobatus tunic*.

Stain	Specific for	Time (min.)	Tunic region	
			TSL*	Common tunic
0.1% toluidine blue in 30% ethanol	Sulfated acid mucopolysaccharide	10-30	++++	++
Aldehyde fuchsin	Sulfated acid mucosubstances	5-15	++	++
Alcian blue pH 2.5	Sulfated acid mucopolysaccharide	14-40	++++	++
Alcian blue pH 1.0	Sulfated acid mucopolysaccharide	15-30	+++	++
Periodic acid-Schiff	Cellulose-like compounds	10	++	++
Sudan black B	Lipid	40	+++	++

*TSL = tunic spicular lamina.

1970; Stiévenart, 1970, 1971). The techniques described by Pearse (1968) for making and using these stains were applied to fresh thin razor blade slices of adult *Cystodytes lobatus* colonies; the results are listed in Table II. Alcian blue and toluidine blue, two stains specific for sulfated acid mucopolysaccharides, stained more heavily around the spicules and inner edge of tunic lining the zooid cavities than elsewhere in the tunic, thus definitely delineating the tunic spicular lamina (TSL). Even those spicules isolated in the tunic during budding and no longer associated with any particular zooids retained this darkly staining membrane around them. (The fact that aldehyde fuchsin did not stain this region in a similar fashion supports Pearse's (1968) statement that it is not the specific stain it has been considered to be).

DISCUSSION

Cystodytes is one of the few genera of ascidians containing mineral concretions that persist in the sediments after the animal's death (Herdman, 1884). In several cases new fossil species of *Cystodytes* have been described solely on the basis of the spicules (Bonet and Benveniste-Velásquez, 1971; Monniot, 1970a; Monniot and Buge, 1971). The taxonomic significance of these spicules depends on the determination of whether or not the spicules form in a species-specific fashion. Monniot (1970b) concluded that polycitorid and didemnid spicules form as a "physico-chemical precipitation of aragonite in the tunic independent of cellular action" and could be used taxonomically only in a general way, to indicate the ascidian group. However, she did intimate that the tunic must play some part in spicule formation due to its fibrous or lamellar structure, but she did not elaborate. The present study proves the existence of an organic matrix for spicule formation (the TSL), differing from the surrounding common tunic in the concentration of sulfated acid mucopolysaccharides. Also, the newly forming spicules have a lumen and are incompletely mineralized (Fig. 9c), while the spicules of adult *C. lobatus* are completely mineralized; this may indicate

another organic matrix for mineralization within each spicule (Lowenstam, personal communication). Cellular action is implicated by the fact that the spicules form in a particular region of the animal, at the junction between abdomen and thorax, and migrate out from that point to the TSL. In addition, a genetic component is implied by the great variation among colonies in the extent of spicule formation. Lafargue and Kniprath's (1978) paper proves the cellular origin of spicules in the Didemnidae. They identified the organ of spicular origin and also found that the spicules are surrounded by a discrete double-layered membrane. Thus their study and this study contradict the findings of Prenant (1925), Pérès (1948) and Monniot (1970b). Pérès was obviously troubled by his inability to determine the reasons for tunic stratification in the Polyclinidae, which he stated was especially noticeable around the periphery of zooids in addition to the thin outer cuticle.

Examination of the spicules in *Cystodytes lobatus* was difficult because nearly any treatment (fixation, sectioning, staining, even relaxation of live zooids) usually resulted in some disruption of the bladder cells and partial dissolution of the spicules. This is why all of the staining was done supravitaly, as suggested by Pearse (1968). Nevertheless, some stains (aldehyde fuchsin and alcian blue pH 1.0 especially) changed the spicules into needle-like clusters within a few minutes. Fixation in alcohol or buffered formalin also disrupted bladder cell membranes, and after being left for a few weeks in fixative the surface irregularities disappeared from many spicules in adult colonies. Indeed, some *Cystodytes* colonies after several years on a museum shelf have few if any recognizable spicules left, a fact which undoubtedly has led to erroneous calculations of the abundance and type of spicules when the colonies were alive (Ritter, 1900; Van Name, 1945; Millar, 1962). Because of these difficulties, taxonomic descriptions of *Cystodytes* and didemnid species should include photographs or at least descriptions of spicules from live colonies if possible.

The question remains of how the tunic spicular lamina can "bud" when the zooid buds, a problem especially puzzling since the lamina appears to be completely extracellular. At a magnification of 675 $\times$, it is possible that a membrane was indistinctly seen in the 1- μ m sections between the outer edge of the tunic spicular lamina and the beginning of the bladder cell region. If this is so, perhaps this membrane somehow separates from the rest of the tunic during budding. It will be necessary to embed and section zooids in the process of budding in order to examine this further.

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SUMMARY

1. The colonial ascidian *Cystodytes lobatus* has a long breeding season (at least 6 months) and releases tadpoles sporadically throughout the day, indicating a long period of recruitment.

2. Tadpoles of *C. lobatus* were settled and reared in the laboratory in order to observe early growth, budding and spicule formation.

3. Budding is preceded by the formation of a new stomach at the posterior end of the esophagus and fits Nakauchi's Type I budding pattern.

4. Spicule formation begins within 5 days after settlement. The spicules appear to form in a particular region at the anterior end of the abdomen and migrate over the abdomen to form a single or slightly overlapping layer embedded in a "tunic spicular lamina." This lamina lies between the common tunic and the zooid cavity and forms a spicular sac in the tunic surrounding but separated from the abdomen of each zooid. It stains especially heavily for sulfated acid mucopolysaccharide; the spicules are concluded to form by cellular action in this organic matrix.

5. There is great variation among zooids in the quantity of spicules formed. These differences are maintained in the buds, resulting in colonies in which all zooids either have few or many spicules, and are therefore probably genetic in origin.

6. During budding the spicular sac becomes disrupted and appears to bud, resulting in a reallocation of the spicules to the buds and formation of separate spicular sacs around the abdomen of each bud. At budding there is apparently no disruption of bladder cell membranes in the tunic and no dissolution of spicules by the acids contained in the bladder cells.

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CHARACTERISTICS AND REGULATION OF FISSION ACTIVITY IN
CLONAL CULTURES OF THE COSMOPOLITAN SEA ANEMONE,
HALIPLANELLA LUCIAE (VERRILL)¹

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The diversity of reproductive modes within the Cnidaria is greater than that of most other metazoan groups. This is particularly evident among the sea anemones, for which Chia (1976) has compiled a list of reproductive modes, and has speculated on their evolution and adaptive significance. Reproduction in *Haliplanella* (= *Diadumene*) *luciae* Verrill is of special interest because *H. luciae* has greatly expanded its geographical range since the turn of the century (Uchida, 1932; Stephenson, 1935; Hand, 1955; Shick and Lamb, 1977). This species now occurs intertidally on boreal (Uchida, 1936; Williams, 1973), temperate (Stephenson, 1935; Hand, 1955) and tropical (Dunn, personal communication; Belem and Monteiro, 1977) coasts.

The ability of *H. luciae* to establish new populations is extraordinary since, unlike other intertidal invertebrates, *H. luciae* has never been observed to produce larvae as agents of dispersal (Davis, 1919; Shick, 1976). All observed reproduction has been asexual, through longitudinal fission (Hargitt, 1912; Davis, 1919), and less commonly by pedal laceration (Atoda, 1954; Johnson and Shick, 1977). Although a single fission event infrequently produces multiple individuals, most fission events are binary, and analogous to cytokinesis (Atoda, 1976; Minasian, 1976).

Longitudinal fission permits the rapid establishment of intertidal clones (Chia, 1976; Francis, 1976). The strategic advantages of fission have been recently discussed by Francis (1976) and Shick and Lamb (1977). Hoffmann (1976) and Shick and Lamb (1977) described the genetic composition of different con-specific clones of anemones, and have made valuable inferences concerning advantages of asexual reproduction in contrast to sexual reproduction.

Understanding the contribution of asexual reproduction to the success of *H. luciae* makes necessary the analysis of asexual reproductive rates and their regulation. However, few studies have evaluated asexual reproductive rates of sea anemones, partly due to a lack of standardized methods.

Researchers of hydroid development have calculated exponential rates of increase (symbolized " k ") in numbers of polyps in laboratory cultures (Loomis, 1954), and have related these rates to environmental variables (Fulton, 1962; Davis, 1971). Minasian (1976) determined k (fission rate) in laboratory populations of *H. luciae*, and quantified the effect of feeding frequency on k . The occurrence of fission in *H. luciae* is affected by temperature (Miyawaki, 1952) and fluctuating

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temperature-emersion treatments (Johnson and Shick, 1977); but the effect of temperature on k was not quantified in those studies.

The present study quantifies the combined effects of temperature and feeding frequency on k in *H. luciae*, and provides a comprehensive analysis of longitudinal-fission activity in a sea anemone. This includes previously undescribed characteristics of fission activity, essential to the understanding of the regulation of longitudinal fission. In addition, we describe a method for establishing and maintaining permanent *in vitro* clonal cultures of *H. luciae*. This has enabled us to obtain a range of possible values of k in a clone of *H. luciae* from northwestern Florida.

MATERIALS AND METHODS

Establishment of clonal cultures

Specimens of *H. luciae* were collected on 2 October from a sand-flat intertidal region adjacent to the Florida State University Marine Lab near Turkey Pt., on the N. W. Florida Gulf coast. These anemones occur on the undersides of sedimentary stones, accompanied by barnacles (*Chthamalus fragilis*) in the upper littoral region, and oysters (*Crassostrea virginica*) in the mid-littoral region. *H. luciae* cloned from this location (29° 54.8' N, 84° 30' W) have a brownish-green column with 12 to 24 yellowish-orange longitudinal stripes in large individuals. Shick and Lamb (1977) found a sample of striped *H. luciae* from this population to consist of two different genotypes (*i.e.* clones). The specimens of *H. luciae* cloned in this study are presumed to belong to the same genotype, since they are phenotypically (sex and color) identical and phenotypically distinguishable from the second striped clone present at the collection site.

To establish clonal cultures, three individual anemones of average diameter (about 5 mm) were isolated in three covered glass storage dishes (Corning #3250), each containing approximately 200 ml natural sea water (28 to 30‰). These cultures were maintained at room temperature (22 to 25° C) under a 14/10 hr light-dark cycle provided by a fluorescent (cool-white) light source, and fed to repletion (30 to 60 min) with newly hatched *Artemia* nauplii every second day. All culture dishes were rinsed briefly with distilled water and refilled with fresh sea water daily. The number of anemones in each culture increased rapidly through longitudinal fission.

After 3 months each clonal culture was divided into two cultures, producing a total of six clonal cultures. These served as stock cultures for the experiments, and were kept under the same photoperiod at a temperature of 16 to 18° C; feeding frequency was reduced to twice per week. Every 4 to 6 weeks each clonal culture was transferred to clean glassware. Clonal cultures were maintained under this standardized regimen for at least 4 months before being used in experiments. Anemones from stock cultures were less than 7 mm in diameter; histological examination showed that none of these anemones bore gonads.

Culture experiments

For each experiment, six duplicate cultures were set up by placing 10 anemones from each stock culture in glass bowls (glass stacking dish, Wheaton #350134)

which were 11.5 cm in diameter, and contained approximately 150 ml of sea water. Sets of six cultures were then placed under one of nine different experimental regimens. These regimens, evaluating combined temperature and feeding effects upon fission activity, were as follows: fed once every 2 days at 26, 21 or 16° C; fed once every 4 days at 26, 21 or 16° C; starved at 26, 21 or 16° C. Thus the entire experiment involved a total of 54 clonal cultures, each initially containing 10 anemones. In addition, fission activity in stock cultures was evaluated by setting up six experimental cultures as above, and observing fission activity for 3 months under maintenance conditions (fed twice per week at 16 to 18° C). Experimental cultures were exposed to the new temperature for 2 days before beginning the experiment, providing time for attachment of anemones to culture bowls. The second day after transfer to culture bowls (day 0) marked the start of the experiment; for experimental groups receiving food, day 0 was the day of the first feeding. These cultures were also under a 14/10 hr light-dark cycle. Anemones were counted daily, at which time they were fed, if necessary, and the sea water changed.

Statistical analysis

Fission rates were calculated from the least-squares regression equation of the natural log number of anemones as a function of time in culture: $\ln N_i = kt_i + \ln N_0$. Here the rate of increase in log number of anemones ($\ln N_i$) on a given day (t_i) is defined by the fission rate (k), which is the slope of the regression line. The number of polyps present at the start (day 0) of the culture interval, and Y-intercept of the regression line, is $\ln N_0$. This equation was employed by Loomis (1954) and Fulton (1962) to calculate the exponential rate of increase (k) for asexual production of hydroid polyps, in which $N_i = N_0 e^{kt_i}$.

Since exogenous factors can impose a variable delay period prior to the initiation of fission, it was necessary to recognize two distinct parameters for fission rate, defined as follows. The overall fission rate (k) is the rate of increase during the entire culture interval, including the initial delay before the onset of fission. The second parameter, k_{adj} , gives the rate of increase subsequent to the initial delay period. Adjusted fission rate (k_{adj}) is calculated by the same method as k , except that the first data entry, day 0, is the day prior to the first occurrence of fission. Hence, k_{adj} designates the rate of increase only after the initiation of fission, rather than during the entire culture period, and better estimates the rate of active, sustained fission. Where the delay before onset of fission is absent or very short, $k = k_{\text{adj}}$. Both k and k_{adj} were calculated for each culture, except where fission activity was not sustained and considered to be nonexponential. The delay before the onset of fission also was calculated for each culture.

The effects of temperature and feeding on k and k_{adj} were evaluated by means of a two-way analysis of variance (ANOVA). Paired comparisons between statistical means were performed using *t*-tests.

The percentage of anemones undergoing fission per day was calculated as follows $(N_i - N_{i-1}) \cdot 100 / N_i$, where N_i is the number of anemones on a given day, and N_{i-1} is the number present on the previous day. An angular transformation was performed on these percentage data, and means and standard deviations

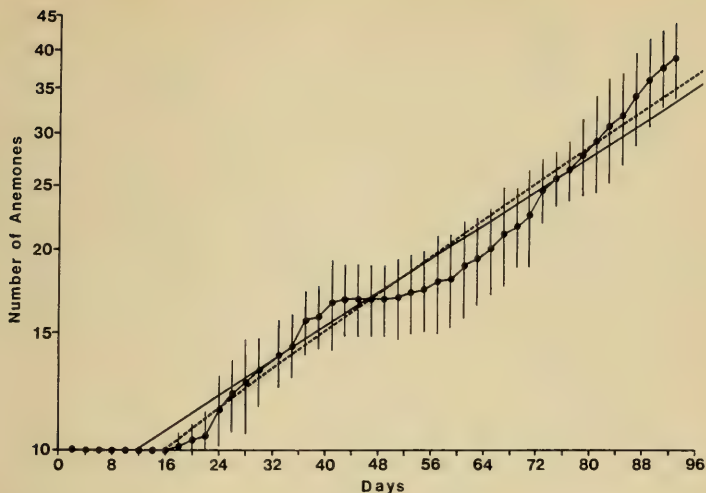


FIGURE 1. Semilogarithmic plot of numerical increase through longitudinal fission in cultures of *H. luciae*, reared under conditions of routine maintenance ($17 \pm 1^\circ$ C, fed twice per week). Day 0 is 2 days after transfer to culture bowls from stock cultures. Each point is a mean of six cultures, each initially containing 10 anemones; error bars are standard deviations. The slope of the regression equation, $\ln N_t = kt_t + \ln N_0$, is the fission rate, k (see Materials and Methods for further explanation). The slope of the solid line (k) is 0.0149 ± 0.0017 (mean $\pm$ s.d.); the slope of the broken line, k_{adj} (0.0162 ± 0.0010), is corrected for the initial delay period prior to the start of fission activity.

calculated for each regimen. On plots of the temporal pattern of fission pulses, the scale of the ordinate conforms to the transformation, whereas the units on the ordinate are actual, untransformed percentages. This transformation is necessary to assume normality (Sokal and Rohlf, 1969). Means and standard deviations were calculated for relative maxima of all fission pulses (pulse maxima), and for relative minimal values between fission pulses (pulse minima); these were evaluated using a multiple-range (Student-Neuman-Keuls) test. Lengths of intervals between fission-pulse maxima were also determined. A G -test for independence (Sokal and Rohlf, 1969) was performed on frequency data for pulse intervals. Test statistics corresponding to probabilities of less than 0.05 were regarded as significant.

RESULTS

Characteristics and exogenous regulation of fission activity

Figure 1 illustrates prominent characteristics of fission activity in clonal cultures reared under the standard maintenance regimen. These characteristics

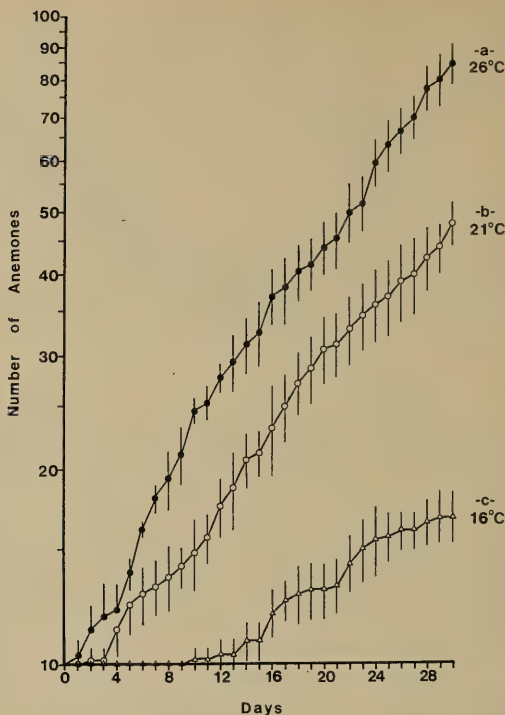


FIGURE 2. Semilogarithmic plot of numerical increase through longitudinal fission in cultures of *H. luciae* fed once every 2 days. Each point represents a mean of six cultures, each initially containing 10 anemones; error bars are standard deviations. Three experimental culture temperatures were used: (a), 26° C; (b), 21° C; (c), 16° C.

included: first, an initial delay period before the onset of fission; second, an exponential fission rate followed by a temporary cessation of fission; and third, resumption of the exponential fission rate. The variable delay lasted for 21.33 ± 4.46 (mean $\pm$ s.d.) days, although one culture initiated fission as early as day 18 (Fig. 1). This delay was initiated by the mechanical disturbance imparted in transferring anemones to new culture bowls. For the entire culture interval of 92 days, $k = 0.0149 \pm 0.0017$ (mean $\pm$ s.d.); with the delay period omitted from calculation, $k_{\text{adj}} = 0.0162 \pm 0.0010$. The calculation for k_{adj} usually increased the estimate of fission rate in addition to shifting the regression line to the right.

Figures 2 and 3 demonstrate the numerical increase in cultures of *H. luciae*

which were fed *Artemia*. At 26° C little or no delay occurred prior to the onset of fission; hence k and k_{adj} were essentially identical. Only when anemones were fed once every two days at 16° C was k_{adj} significantly larger than k (t -test, $P < 0.05$).

Values of k_{adj} in experimental cultures ranged from 0.0278 (doubling time = 24.9 days) at 16° C to 0.0728 (doubling time = 9.5 days) at 26° C, in cultures fed every second day. Analyses of temperature and feeding effects showed both factors to significantly affect k and k_{adj} , based upon comparisons between 21° and 26° C regimens (Table I). Temperature coefficients (Q_{10}) for k_{adj} over the 21 to 26° C temperature range were 1.6042 and 1.6759 for feeding frequencies of 2 and 4 days, respectively (Table II). Thus, the difference in feeding frequency did not influence the effect of temperature upon k_{adj} in this range. Since the Q_{10} for the 16 to 21° C range was much larger (Table II), the greatest effect of temperature on k_{adj} occurred below 21° C.

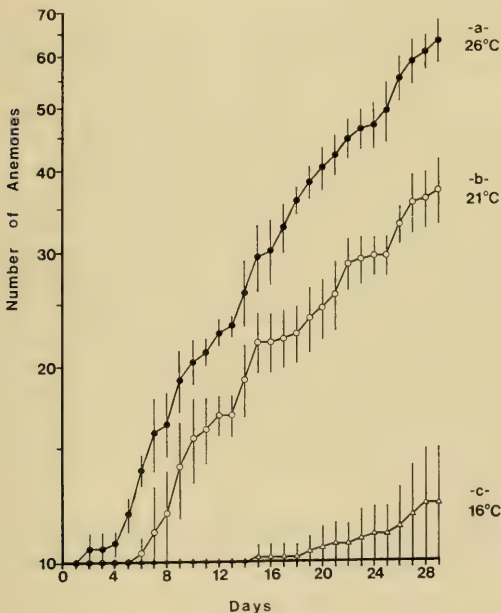


FIGURE 3. Semilogarithmic plot of numerical increase through longitudinal fission in cultures of *H. luciae* fed once every 4 days. Each point represents a mean of six cultures, each initially containing 10 anemones; error bars are standard deviations. Three experimental culture temperatures were used: a), 26° C; b) 21° C; c), 16° C.

TABLE I

Means $\pm$ standard deviations for values of k and k_{adj} for *H. luciae* reared at different temperatures and feeding frequencies. Each experimental mean consisted of six bowls, each initially containing 10 anemones. The resulting two-way ANOVA was performed on four means (21° vs. 26° C, fed every second or fourth day). The ANOVA results were the same for both k and k_{adj} , and indicate if an effect upon k was statistically significant (+) or not significant (–) at the 5% level; n.c. = fission activity not sufficient to calculate k .

Feeding frequency	Temperature		
	16 $\pm$ 1° C	21 $\pm$ 1° C	26 $\pm$ 1° C
Values of k			
2 days	0.0207 $\pm$ 0.0036	0.0569 $\pm$ 0.0040	0.0728 $\pm$ 0.0035
4 days	n.c.	0.0518 $\pm$ 0.0031	0.0695 $\pm$ 0.0032
Values of k_{adj}			
2 days	0.0278 $\pm$ 0.0047	0.0574 $\pm$ 0.0035	0.0727 $\pm$ 0.0036
4 days	n.c.	0.0533 $\pm$ 0.0045	0.0690 $\pm$ 0.0025
ANOVA: Effects for 21° vs. 26° C			
Temperature	Feeding		Interaction
(+)	(+) (+)		(–)

Both temperature and feeding significantly affect length of the delay period prior to the initiation of fission (Table III). The duration of delay periods varied from 1 day or less at 26° C, to over 21 days at 16° C. Table III shows that decreasing the feeding frequency from 2 to 4 days further lengthened this delay. Temperature and feeding frequency acted synergistically upon the pre-fission delay, as indicated by the significant ANOVA interaction term (Table III). For example, at 26° C halving the feeding frequency (from every second to every fourth day) increased the mean delay period by 1.3 days; at 16° C, halving the feeding frequency increased the delay by over 10 days (Table III).

Fission activity in starved cultures at 26° C and 21° C was limited to two major pulses of fission activity (Fig. 4); starved anemones at 16° C did not

TABLE II

Temperature coefficients (Q_{10}) for fission rate (k_{adj}) in *H. luciae* at two different feeding frequencies; n.c. = insufficient data for calculation.

Feeding frequency	Temperature Range		
	16°–21° C	21°–26° C	16°–26° C
2 days	4.3567	1.6042	2.6436
4 days	n.c.	1.6759	n.c.

TABLE III

Means $\pm$ standard deviations for delay to onset of fission (days) in *H. luciae* at different temperatures and feeding frequencies. Each experimental mean consisted of six bowls, each initially containing 10 individual anemones. The two-way ANOVA result indicates if an effect on fission delay is statistically significant (+) or not significant (–) at the 5% level.

Feeding frequency	Temperature		
	16 $\pm$ 1° C	21 $\pm$ 1° C	26 $\pm$ 1° C
2 days	11.6667 $\pm$ 2.3381	1.8333 $\pm$ 0.9832	0.0000 $\pm$ 1.0954
4 days	>21.0	5.5000 $\pm$ 1.3784	1.3333 $\pm$ 1.5055

ANOVA: Effects for 21° vs. 26° C

Temperature	Feeding	Interaction
(+)	(+)	(+)

undergo fission. Nonetheless, these cultures remained healthy throughout the experiment.

Temporal pattern of fission activity

After long delay periods a major pulse of synchronous fission occurred, followed by a temporary cessation of fission (Figs. 1, 2c). The resumption of fission after this initial pulse was either less synchronous and without additional major plateaus (Fig. 1), or continued to show additional, brief cessations of fission (Fig. 2c).

H. luciae exhibited distinct pulses of increased fission activity, with relative maxima (peaks) and minima (troughs), shown in Figures 5 and 6. These occurred

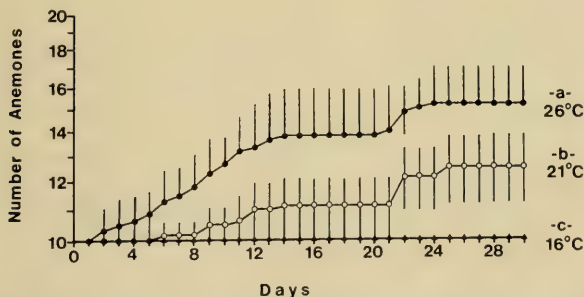


FIGURE 4. Semilogarithmic plot of numerical increase through longitudinal fission in starved cultures of *H. luciae*. Each point represents a mean of six cultures, each initially containing 10 anemones; error bars are standard deviations. Three experimental culture temperatures were used: (a), 26° C; (b), 21° C; (c), 16° C.

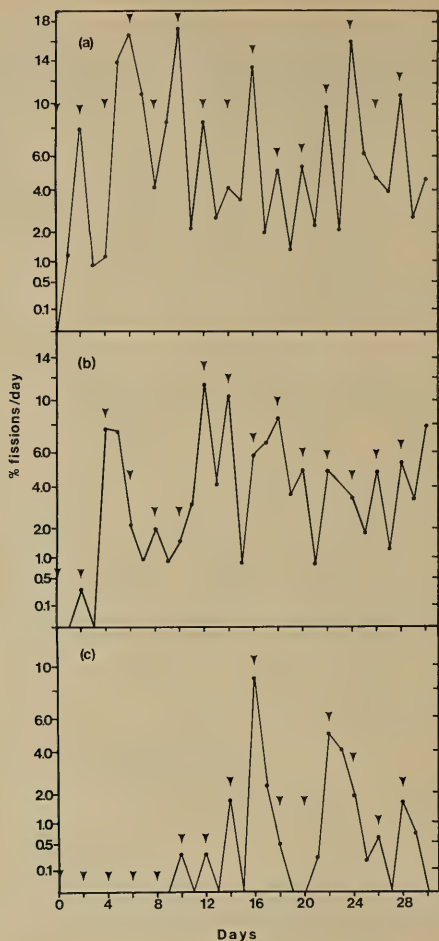


FIGURE 5. Percentages of *H. luciae* undergoing fission per culture per day in cultures fed once every 2 days. Each percentage is a mean of six cultures; arrowheads indicate feeding days. Data was subjected to angular transformation prior to statistical computations; plotted means are transformed data. The ordinate indicates actual, untransformed percentages. Three culture temperatures are represented: (a) 26° C; (b) 21° C; (c) 16° C.

TABLE IV

Different lengths of time periods between peak values of fission activity (pulse maxima), observed in H. luciae culture populations. Cultures initially consisted of 10 anemones each and were reared under different temperature and feeding regimens for 1 month. Six clonal cultures were reared under each different culture regimen. The percentage of fissions occurring each day were then averaged, and the peaks of fission activity determined. Occurrence of the various lengths of pulse intervals depend upon culture conditions (G-test for independence, $P < 0.05$).

Culture regimen	Length of period between pulse maxima (days)			
	2	3	4	>4
26° C, fed every 2 days	7	0	3	0
26° C, fed every 4 days	0	5	1	1
21° C, fed every 2 days	5	0	4	0
21° C, fed every 4 days	1	3	2	0
16° C, fed every 2 days	4	0	1	1

in cycles, with intervals between pulse maxima usually lasting for 2 to 4 days. These pulses of fission had a phasic dependence upon the feeding regimen. In cultures which were fed every second day, pulse maxima occurred only on feeding days, although not on all feeding days (Fig. 5). Therefore, periods between pulse maxima were multiples of 2 days, in cultures fed every second day. At 26° and 21° C most pulse maxima had a 2-day periodicity, with fewer pulse maxima being 4 days apart (Fig. 5a, b). In the 16° C cultures, a 6-day period between pulse maxima occurred at one point, although 2-day periods were still most frequent (Fig. 5c).

In cultures fed at 4-day intervals pulse maxima often occurred on days other than feeding days, and were usually 3 or 4 days apart (Fig. 6). Thus, the periodicity of pulse maxima was longer than that in cultures receiving food at

TABLE V

Means and standard deviations for fission-pulse maxima and minima observed in cultures of H. luciae reared under different culture regimens. Each determination was taken from angular-transformed, averaged data from six clonal cultures. Means are for pulse maxima or minima occurring over a one-month period. Sample sizes are in parentheses. Asterisks denote means which are significantly different from all other means in the same column (Student-Neuman-Keuls test, $P < 0.05$).

Culture regimen	Pulse maximum†	Pulse minimum†
26° C, fed every 2 days $k_{adj} = 0.0727$	18.359 ± 4.601 (11)	8.823 ± 1.893 (11)*
26° C, fed every 4 days $k_{adj} = 0.0690$	16.967 ± 5.039 (8)	6.486 ± 3.790 (8)
21° C, fed every 2 days $k_{adj} = 0.0574$	13.393 ± 5.014 (10)	6.828 ± 3.467 (10)
21° C, fed every 4 days $k_{adj} = 0.0533$	15.602 ± 7.650 (8)	3.658 ± 3.226 (8)*
16° C, fed every 2 days $k_{adj} = 0.0278$	7.920 ± 5.390 (7)*	0.417 ± 1.021 (6)*

† angular transformation ($\arcsin\sqrt{\% \text{ fissions/day}}$) is necessary to assume normality.

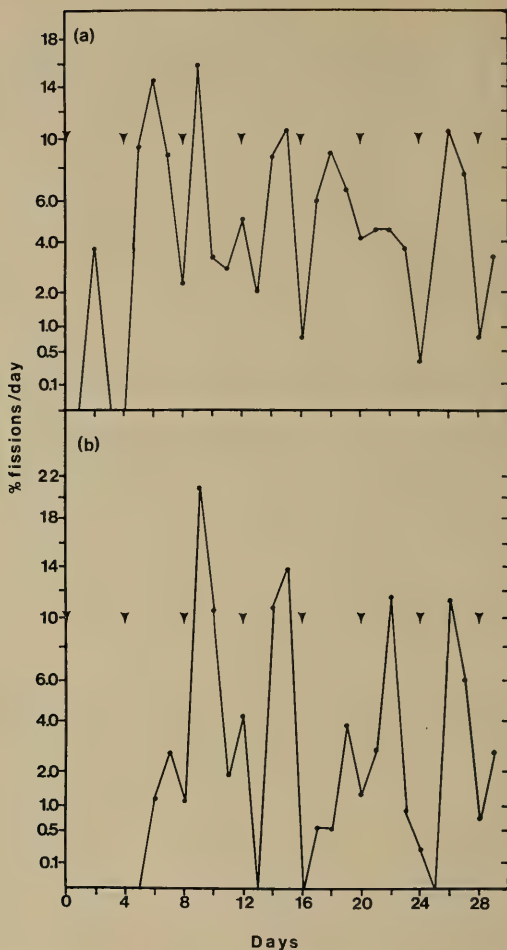


FIGURE 6. Percentages of *H. luciae* undergoing fission per culture per day in cultures fed once every 4 days. Each percentage is a mean of six cultures, each containing 10 or more anemones; arrowheads indicate feeding days. Data was subjected to angular transformation prior to statistical computations; plotted means are transformed data. The ordinate indicates actual, untransformed percentages. Two culture temperatures are represented: (a) 26° C; (b) 21° C.

2-day intervals. Only one period of 2 days was observed at the 4-day feeding frequency (Fig. 6b). Table IV summarizes the occurrence of pulse periodicity, which is dependent upon culture conditions (G -test, $P < 0.05$).

Pulse maxima often attained 10 to 20% of the culture population undergoing fission per day (untransformed percentages are given on the ordinate in Figs. 5 and 6). Pulse minima usually dropped below 4% fissions per day in cultures exhibiting lower values of k (Table V). Mean values of pulse maxima were generally indicative of values of k_{adj} . For example, a mean pulse maximum of 13.393 ($\arcsin\sqrt{\% \text{ fissions/day}}$) corresponded to a mean k_{adj} of 0.0574, while a significantly lower (Student-Neuman-Keuls, $P < 0.05$) mean pulse maximum of 7.920 ($\arcsin\sqrt{\% \text{ fissions/day}}$) accompanied a k_{adj} of 0.0278 (Table V). Likewise, significantly lower values of k_{adj} accompanied lower values for pulse minima (Table V).

DISCUSSION

The distinction between k and k_{adj} is important for the analysis of fission activity in *H. luciae*. Fluctuations in population size and density are best considered in terms of k rather than k_{adj} , since periods of fission delay, as opposed to active periods of fission, are not easily distinguished in field studies. Moreover, fission-related morphological variability (Minasian, 1979) is best understood in relation to the total number of fissions over an entire interval (k), rather than the sustained or maximal rate of fission during part of that interval (k_{adj}). The parameter k_{adj} is a more accurate indication of active fission than is k . Hence, k_{adj} should be used to examine experimental effects upon fission rate. The calculation of k_{adj} requires that data be collected at frequent, preferably daily intervals.

The present study reveals three different effects of temperature upon fission activity. The first effect is upon fission rate. The Q_{10} value for k_{adj} in the 16 to 21° C range was three times greater than the Q_{10} value for the 21 to 26° C range. Sassaman and Mangum (1970) similarly found that oxygen consumption in *H. luciae* showed disproportionately large Q_{10} values over a range of 10 to 17.5° C. The second effect of temperature, a lengthening of the delay period, was also greatest below 21° C. Temperature also affected fission-pulse patterns: in cultures fed every second day, a 10° C decrease in culture temperature caused a 40% drop in the frequency of fission pulses, and a significant decrease in values of pulse maxima and minima.

The great response of fission activity to small changes in temperature indicates that temperature is the foremost exogenous regulator of k . Miyawaki (1952) believed that a temperature threshold of approximately 15° C, below which no fission occurred, existed for *H. luciae*. He observed no longitudinal fission at temperatures below 20° C. The present study shows that values of k for this Florida clone decrease sharply as temperatures descend to 15° C, and in this respect agrees with Miyawaki's (1952) observations on Japanese *H. luciae*. However, a temperature threshold which limits fission in *H. luciae* is dependent upon other parameters which also affect k , such as food availability. Thus, k is best regarded as having a graded response to temperature, rather than an absolute threshold of 15° C.

The effect of feeding frequency upon k appears to be temperature-dependent. Although halving the feeding frequency resulted in reductions of k by only 4.5% at 26° C, and 8.9% at 21° C, a relatively large effect of decreased feeding frequency upon k occurred at 16° C, where the delay period was greatly lengthened. A temperature-dependent effect of feeding on the morphology of *H. luciae* reflects such interaction of effects on k (Minasian, 1979).

Exogenous factors determine sensitivity to mechanical stimuli which interrupt fission activity. Transferring anemones to new culture vessels (when setting up experimental cultures) imparted a mechanical disturbance, which caused little or no fission delay at high temperature and feeding frequency; but at low temperatures and feeding frequencies this delay may last for several weeks. Johnson and Shick (1977) demonstrated that fluctuating immersion-emersion cycles decrease fission activity in *H. luciae*. The mechanism underlying this effect may involve mechanically induced delays.

The synchrony of fission cessation following initial fission activity in low-temperature cultures (Fig. 1) is reminiscent of a synchronized cell culture (*c.g.*, Zeuthen and Scherbaum, 1954; Zeuthen, 1964), and implies the existence of relatively constant periods between fissions among individuals within the culture population. This long-term fission synchrony is most evident at low temperatures because periods between fission events are lengthened. When exogenous factors permit only short periods between fissions, such long-term synchronization is not observed. Studies on the lengths of inter-fission periods must be made on individual anemones to ascertain if inter-fission periods are relatively constant in duration, and how they change due to exogenous influences. The inter-fission period may simply be a delay in response to the previous fission event, which itself constitutes a mechanical disturbance.

The coincidence of fission pulses with feeding days, in cultures fed every second day, points to a regulatory role for other exogenous, and possibly endogenous factors. Photoperiodic and feeding-digestion cycles appear to be involved. For example, cultures of *H. luciae* are more active during dark portions of the photoperiodic cycle, and usually exhibit greater fission activity in the dark (Minasian, unpublished data). Similarly, Batham and Pantin (1950) observed pedal locomotory activity of the sea anemone, *Metridium senile*, to occur only at night. Although feeding causes long-term enhancement of fission, Torrey and Mery (1904) suggested that fission in *H. luciae* is inhibited by feeding. Thus, interaction of photoperiodic rhythms and short-term inhibition by feeding produces a 2-day pulse pattern in cultures fed at 2-day intervals: fission is inhibited during the first dark period subsequent to feeding, followed by a release from inhibition and increased fission activity during the second dark period after feeding. This inhibition of fission during hours subsequent to feeding explains the even-day duration of periods between fission-pulse maxima in cultures fed at 2-day intervals.

In cultures fed at 4-day intervals, periods between fission-pulse maxima are longer, with pulses of fission activity often beginning during the 24 hr subsequent to feeding. Minasian (1976) similarly observed that *H. luciae* undergo more synchronous fission activity in response to renewed feeding when starved for longer periods. Thus, cultures fed at different frequencies have inherent differences in the response of fission activity to feeding. If there exists an endogenous influence

on the fission-pulse pattern, it may permit synchronization of fission pulses with 2-day feeding intervals, but not with 4-day intervals.

The extreme sensitivity of fission activity to temperature indicates that the clone of *H. luciae* examined in these culture experiments must achieve recruitment primarily when temperatures exceed 20° C. Below 20° C, low-temperature inhibition is reinforced by tidal cycles, which can limit food availability and impart mechanical disturbance through immersion-emersion effects. Hence, below 20° C k will be small, and delays between major pulses of fission activity will be long. Previous studies (Shick and Lamb, 1977; Minasian 1979) have stated that the absence of gametogenesis (*i.e.*, absence of sexual reproduction) in *H. luciae* is associated with small size (and hence high k). Thus, the 15° to 20° C temperature range probably marks a crucial transition from small, sterile and strictly asexual anemones exhibiting high k , to larger, sexually reproductive anemones which exhibit only infrequent fission. Under present culture conditions, periodic, pulsed increments of fission activity are best interpreted in relation to two periodic, exogenous stimuli: photoperiod and feeding. In natural populations of *H. luciae*, periodic stimuli include photoperiod and tidal fluctuation, of which the latter involves both feeding and a mechanical (immersion-emersion) effect (Johnson and Shick, 1977). Therefore, it is possible that such pulses of fission activity have a phasic dependence upon tidal and photoperiodic cycles in intertidal populations of *H. luciae*. An investigation of fission activity under field conditions may elucidate this relationship.

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SUMMARY

1. Permanent cultures of a clone of *H. luciae* from N. W. Florida were reared under different temperature and feeding regimes in order to identify and quantify parameters of asexual reproduction.

2. The principle components of fission activity include fission rate, a delay period following a mechanical disturbance, and periodic pulses of increased fission activity; all components are regulated by temperature and feeding frequency.

3. A distinction is made between fission rate including the delay period (k), and fission rate following the delay period (k_{adj}).

4. Fission rates (k_{adj}) ranged from 0.0162 (doubling time = 42.8 days) at 17° C to 0.0727 (doubling time = 9.5 days) at 26° C.

5. Temperature is the foremost regulator of k ; the greatest influence of feeding frequency was upon periodic pulses of fission activity.

6. Culture data indicate that recruitment in natural populations of this clone is restricted by seasonal temperature; below 20° C there is a sharp reduction in k . It is suggested that inhibition of k by temperatures below 20° C favors a transition from asexual to sexual reproduction.

7. The pulsatile, periodic character of fission activity is prominent in laboratory cultures, and suggests that such activity in natural habitats may have a phasic dependence upon tidal and photoperiodic cycles.

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LOCOMOTOR AND LIGHT RESPONSES OF LARVAE OF THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS* (L.)

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The American horseshoe crab, *Limulus polyphemus* is an abundant and conspicuous member of the estuarine fauna of the Atlantic coast of the United States and the Gulf of Mexico. Its importance as a laboratory animal for neurophysiological, biochemical and medical research is well established (Wolbarsht and Yeandle, 1967; Cohen, 1979). Despite these facts, remarkably little has been recorded on the ecology and natural history of the species. The limited information available (Shuster, 1958; Sokoloff, 1978; Rudloe, 1979; Rudloe, 1978; Rudloe and Herrnkind, 1976) has primarily been concerned with the adult phase of the life cycle, especially the emergence of breeding crabs onto sandy beaches.

The juvenile and larval phases of the life cycle are of equal interest, however. Juveniles are important predators of the sandy intertidal community in areas adjacent to adult breeding beaches (Green and Hobson, 1970; Rudloe, 1978), while the resemblance of the larval instar to certain trilobites has long been a source of comment and is sometimes cited as an indication of the phylogenetic antiquity of the species.

Larvae hatch approximately 5 weeks after the female lays her eggs near the high tide mark. The time may vary depending on ambient temperature, and other environmental factors (Jegla and Costlow, 1979). The embryological development of *Limulus* eggs is well described by Kingsley (1892). However, field studies of this phase of the life cycle are limited to observations by Shuster (1958) on nematode, oligochaete and maggot activity in the nests, and Hummon, Fleeger and Hummon (1976) who described the interaction of beach meiofauna with developing eggs.

Newly laid eggs are sticky and occur as tightly clumped balls. When the eggs hatch, the larvae remain in distinct aggregations at depths comparable to those of newly laid eggs. The larvae eventually reach the surface of the sand and emerge into the water column. When larvae emerge from the nest or when they are removed and exposed to water, they exhibit a "swimming frenzy" reminiscent of neonate sea turtles, swimming vigorously and continuously for hours. They are also, like the larvae of many other marine invertebrates, strongly positively phototactic, orienting immediately to any available light source (Thorson, 1964; Rudloe, 1978). Loeb (1893) first mentioned the response of *Limulus* larvae to light, while the development of the swimming behavior has been described by Pearl (1904) as a continuum from embryo to larvae. More recently, French (1977) and French and Doliner (1978) have also described aspects of larval light responses in the laboratory.

While juvenile and adult horseshoe crabs are very similar in morphology, the larval (post hatching) instar is morphologically specialized and unlike all sub-

sequent stages. This larval instar is confronted with the necessity of moving successfully from a nest buried several centimeters beneath the sand surface at a high level of the beach out into the marine environment. Once this transition is achieved, the animal will not re-enter the upper beach environment until it is a sexually active adult. At that time it will be vastly changed, both behaviorally and morphologically, from the larval stage. The timing and mechanisms of release of larval specimens of *Limulus*, as well as the characteristic behavioral patterns of the larvae that contribute to achieving this movement away from the nest and which differentiate it from other phases of the life cycle were the objectives of this study.

MATERIALS AND METHODS

Field studies

Activity of larvae was monitored at Mashers Sands Beach, Wakulla County, Florida from May through November, 1977, June and July, 1978, and in June, 1979. Nests in this area occurred in a distinct zone at the level of extreme high tide and could be easily located by digging at that height on the beach. A series of nests was dug open and the depth from the center of the nest to the surface was measured at periodic intervals ranging from 3 to 7 days between May and November, 1977, for a total of 21 samples. The number of nests measured on each date varied from 10 to 25. Digging was always done within three hours of the daylight high tide.

In addition, the depths of six nests chosen haphazardly each hour were checked over an 11-hr period on the night of full moon in June, 1978. Different nests were measured at each hour, for a total of 66 nests. The nests were dug open by hand and the depth from the center of the larval aggregation to the surface was measured. Nests remained tightly packed after hatching so that accurately locating the center was not difficult. Sampling was also done at the hour of high tide at seven-day intervals thereafter to ascertain nest depths at high tides on the following new moon spring tide and at the following two neap tides. Hourly sampling of six nests was conducted on the night of new moon in June, 1979, using the method described above. Nests were checked from low tide until the hour of high tide, for a total of 48 nests.

Surface plankton tows, each of 4-min duration, were made parallel to the beach at a depth of 1 m for day and night high tides on 128 tides between May and November, 1977, using a coarse mesh net constructed of wire window screening. This mesh retained the macroscopic *Limulus* larvae while passing smaller zoo- and phyto-plankters. The captured larvae were counted and released.

Laboratory analysis of locomotor activity rhythms

Larvae were examined in the laboratory for locomotor activity rhythms using time-lapse photography. An automatically triggered motordrive 35-mm camera using black and white negative film was used, as was an 8-mm movie camera set for timed exposures. The still camera recorded a frame once every 20 min, whereas the 8-mm camera recorded every 2 min. In all cases the data were grouped into 1-hr intervals.

Activity was recorded under various light regimes. Ambient light (with care taken to insure that no artificial light source was present) with an approximate light regime of fifteen hours of light and nine hours of darkness (LD) and water temperatures ranging between 28° C and 34° C was used to approximate conditions of light and temperature encountered in the upper intertidal zone. Constant light and temperature (LL) and constant darkness and temperature (DD) were also used.

Five hundred larvae were used in each of three DD trials, and 1000 larvae were used in all other trials. These densities did not result in any significant mortality, but, assessed visually, did approximate concentrations observed in the field. Larvae were collected from the field prior to each trial on the nights of full moon, new moon, and at 7-day intervals before and after (the lunar quarters), by digging nests of hatched but unreleased animals. All trials were run until the larvae molted into the morphologically distinct first juvenile instar, usually for 6 to 8 days. Animals held under DD did not molt this rapidly and were tested for 12 days.

An additional four sets of 1000 larvae were tested for 1 week each over 1 lunar month, from full moon to full moon in August, 1978. Trials were initiated on the nights of full moon, full moon plus 7 days, new moon, and new moon plus 7 days, and continued until molting of the larvae as above. Larvae were collected from the field 8 hr prior to the start of each trial, by digging nests of hatched but unreleased animals. An ambient light and temperature regime was employed, and activity was recorded as described above, using time-lapse photography. Lunar variability in the positive response of larvae to a light source was also tested during these trials by exposing the larvae to a constant light gradient during the hours of darkness. This light was provided by an overhead source (Tensor Hi Intensity Lamp #C3812), at a distance of 1 m from the water surface. This illuminated 25% of the test aquarium with white light. The remainder of the aquarium was shaded from the light with an opaque cover, creating a steep gradient of light intensity between the lighted quadrant and the rest of the aquarium.

All trials were conducted in 20-gal glass aquaria with a sand substrate into which animals buried when they were inactive. A closed system of circulation was employed with subsand filtration. Temperatures ranged from 28° to 34° C during the ambient light regime trial and were held at 24° C under constant light and darkness. Salinity was held at 20‰ in all trials.

RESULTS

Field studies

Nest depths in the field showed no consistent change from the beginning to the end of the 8-month breeding season. The mean depth for larvae (13.2 ± 2.9 cm) was not significantly different than that for newly laid and immobile eggs (14.6 ± 1.9 cm) although the standard deviation was somewhat larger ($t = 1.25$, n.s.). However, there was a pronounced short term movement of larval nests upward from a mean depth of 17 cm at low tide to the surface 6 hr later at high tide on the night of full moon (Fig. 1). This was followed by movements of the larval nests back down to depths of approximately 14 cm by the time of the next low tide. Nests could be observed at the surface for 30 min prior to and 30

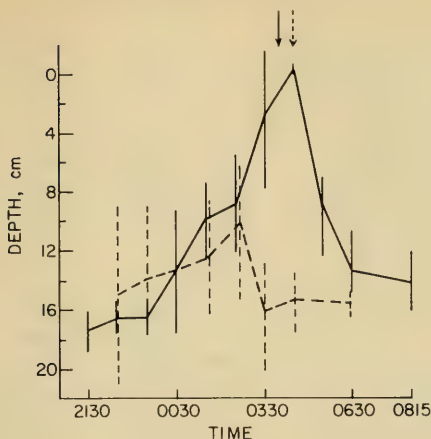


FIGURE 1. Movement of larval nests to and away from the surface of the beach during nights of full moon (solid line) and new moon (dotted line). The arrow indicates the time of high tide in each case. Bars indicate 1 s.d. Movement of nests to the surface on the night of full moon was not repeated on the night of new moon.

min after the high tide. Usually, some 15 to 20 individual larvae become partially exposed at the surface, with the rest of the nest remaining immediately below the surface. Each wave washed away the surface animals and carried them down the beach into the water. Many were cast back up onto the beach and stranded as the tide receded, where they formed drift lines of larvae on the beach. Most of these animals remained at the surface until daylight, after which they disappeared, falling prey to shore birds at dawn. The willet, *Catoptrophorus semipalmatus*, was a major predator. Reburial was probably not of great significance since scattered individuals were only occasionally found beneath the surface. Thus, larvae were seen to move in the field from their original burial depths to the surface and back down again over a 12 hr tidal cycle on the night of full moon. Such pronounced movements to the surface did not occur on the night of new moon. There was no significant difference between mean nest depth at low tide and mean nest depths at high tide on the night of new moon in 1979.

The depths of nests at the hour of high tide on the subsequent neap tide, new moon spring tide and second neap tide of the lunar month in 1978 are presented in Figure 2. Only on the night of the full moon spring tide did larvae come to the surface. The water did not reach the level of the nests at any time other than during spring tides at full and new moon.

The results of the plankton sampling adjacent to the breeding beach on day and night tides throughout the breeding season are provided in Figure 3. The major releases occurred on the night of full moon with two exceptions. And, as with breeding adults (Rudloe 1978), a strong nocturnal activity rhythm is evident.

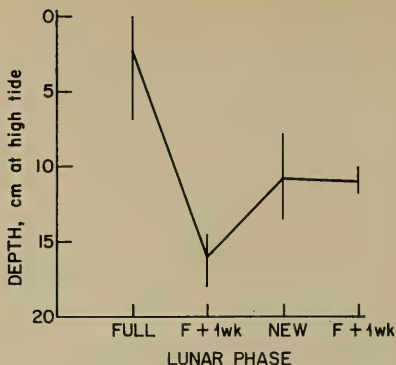


FIGURE 2. Mean depth of larval nests at the hour of high tide at each lunar phase for one month. Bars indicate 1 s.d.

There was also a major release of larvae whenever localized storms with strong onshore winds coincided with high tide, producing an unusually heavy surf. Under these circumstances, larvae were released by day as well as by night. Following a massive storm release in July, the following full moon did not show the usual peak of emergence. The larvae that would have emerged then may have been washed out in the preceding storm. Thus, the occurrence of storm conditions that coincide with a sufficiently high tide are an additional release mechanism.

Laboratory studies

When larvae were maintained under ambient light and temperature regimes, they displayed strong nocturnal activity peaks for the duration of the larval instar (Fig. 4). Larvae remained buried below the surface of the sand substrate during daylight hours. The abruptness with which activity appeared at the same hour each evening and terminated at the same hour each morning suggests that activity might be triggered very precisely by some as yet undescribed factor such as light intensity.

That the nocturnal rhythmicity of behavior might be endogenous in nature is suggested by the results of the trials in which activity was recorded under constant conditions of darkness and temperature. In Figure 5 larval activity is seen to occur during the hours corresponding to normal darkness almost exclusively. It was also noted that none of the larvae used in these trials molted as rapidly to the first juvenile instar as did those maintained under ambient light and temperature conditions.

When maintained under LL conditions and constant temperature, the larvae sustained slight nocturnal activity peaks for the first 3 days (Fig. 6) of the trial, although the amplitude of the variation was considerably reduced, and substantial numbers of larvae remained active during daylight hours. Overall activity was well

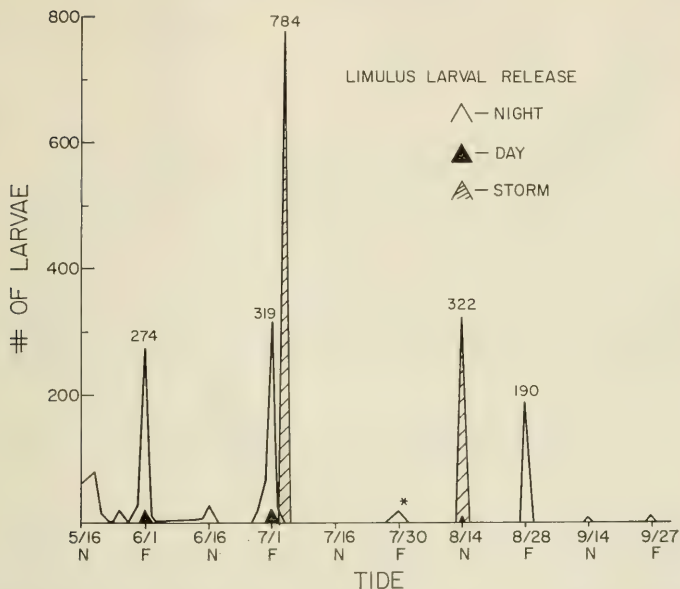


FIGURE 3. Appearance of free-swimming larvae in plankton samples adjacent to a breeding beach throughout a breeding season. F = full moon; N = new moon. Open peaks represent night high tides, closed peaks represent day high tides, and shaded peaks represent storm release. The asterisk denotes the July full moon, when larval release was not significant. This may be due to the extreme release of larvae on the preceding full moon when a massive storm release coincided with the lunar release.

below that under DD and ambient light regimes, however, and reached a level of zero by the fifth day of the trial. When light was removed for 30 min on the fifth night, swimming activity resumed within 5 min of the onset of darkness. After 30 min of darkness, light was restored and activity showed a steady decline throughout the rest of the night. This experiment was terminated thereafter due to the molting of the larvae.

A comparison of levels of activity and light responses of the larvae tested for 7 days each for each week of a lunar month are presented in Table I. Larvae were more active during weeks of spring tides (new and full moon) than on the intervening weeks of neap tides ($t = 5.49$, $P < 0.01$). Differences in levels of activity for the nights of new and full moon in the laboratory were not significant, however.

Although there was no significant difference in over-all first night activity between new and full moon, there was a marked difference in the pattern of activity

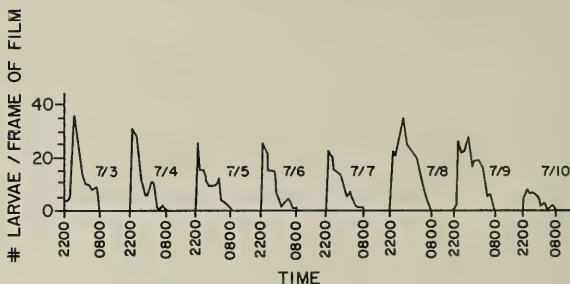


FIGURE 4. Activity patterns of 1000 *Limulus* larvae maintained in the laboratory under conditions of ambient light and temperature. Swimming activity began at 2200 hr each night and terminated at 0800 hr each morning. Larvae remained buried in the sand substrate when not actively swimming. Data is present as number of larvae visible in 1 frame of film at each hour.

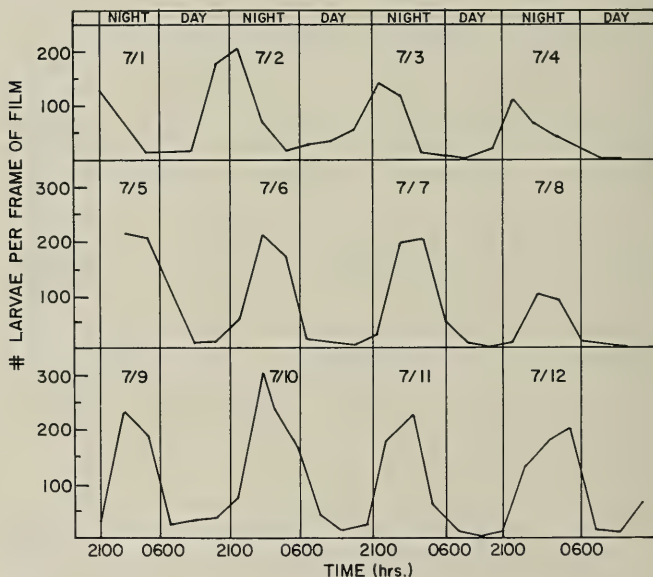


FIGURE 5. Swimming activity of 500 *Limulus* larvae maintained in the laboratory under conditions of constant temperature and darkness. One trial is presented with a duration of 12 days, after which the larvae molted.

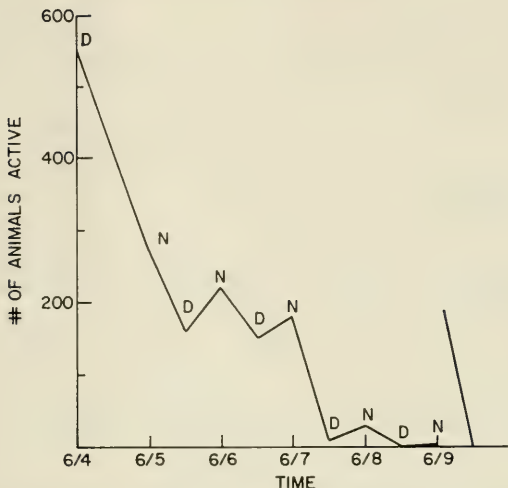


FIGURE 6. Swimming activity of 1000 *Limulus* larvae maintained in the laboratory under conditions of constant temperature and light. Activity terminated on the fifth night but was resumed immediately when light was removed for 5 min at 0100 hr. With restoration of the light, activity again declined. Animals molted during the next 24-hr period and the experiment was terminated. D is daylight at 1200 hr, N is night at 2400 hr. Number of animals swimming was plotted hourly.

during these nights. During the course of the full moon night, activity increased by a factor of 114% between 10 PM and 7 AM while it declined by 29% during the same period during the course of the new moon night. It declined even more steeply, by factors of 79% and 81% on the nights of neap or quarter moon tides. Activity also declined steeply from first to last nights of the session for the full moon (82%) and neap tide (78, 89%) weeks, but declined less sharply from first to last night during the new moon week (30% decline in activity).

Larvae were significantly attracted to light in all lunar weeks (Table I) except that of the new moon. Although larvae were active and swimming on the night of new moon, they showed no significant response to light as measured by concentration of individuals in the lighted quadrant of the aquarium. This lack of responsiveness was maintained throughout the entire week of the new moon trial.

DISCUSSION

The use by *Limulus* of the intertidal sandy beach as a breeding site and the burial of nests well below the surface affords a great deal of protection for developing eggs, and most survive to hatching. The occurrence of larval nests in a distinct zone in the upper intertidal in the northeastern Gulf of Mexico may be related

TABLE I

Lunar variation in larval swimming activity and light responses under laboratory conditions. Four sets of 1,000 larvae, one for each lunar week, were tested. The larvae responded to light at all times other than the week of new moon.

	Lunar phase			
	Full	New	Full - 1 week	Full + 1 week
Mean activity, first night	580	485	329	393
% decline in activity, first night	(increased)	29	79	81
% decline in activity, first to sixth night	82	30	78	89
% responding to light, first night	63	31	78	89
% decline in light response, first night	31	—	38	57
% decline in light response, first to sixth night	32	—	(increased) 113	38
X ² , light response, first to sixth night	49.8	9.44	49.8	64.6
	$P < 0.01$	N.S.	$P < 0.01$	$P < 0.01$
X ² , light response, first night	246	6.52	69.5	131.7
	$P < 0.01$	N.S.	$P < 0.01$	$P < 0.01$

n = 1000 in each trial.

to the small tidal amplitude of approximately 1 m. According to Shuster (personal communication), nests are found in Delaware Bay, where the spring tide range is approximately 2 m, over a much wider band—from the high tide line through 60% of the exposed intertidal. In Cape Cod Bay, where the range is 3 m or more, nests are also widely distributed but tend to be most numerous in the mid tidal area.

In addition to protection, this nesting pattern also creates substantial problems of emergence and dispersal of the young into the aquatic environment. However, *Limulus* larvae are a behaviorally and morphologically specialized phase of the life cycle, that cope well with the problems of nest release. With an initial swimming frenzy and positive phototaxis, and with lunar and circadian activity rhythms that synchronize activity with water levels on the beach, the larvae are well adapted for emergence from the nests and dispersal. After this transition is achieved, larvae molt into the substantially different morphology and behavior patterns characteristic of the benthic portion of the life cycle.

Although further work is required to be conclusive, the nocturnal swimming of *Limulus* larvae under DD conditions suggests an endogenous rhythm. The peaks of activity appear to occur progressively later during the night as the experiment progresses, and these peaks are approximately associated with the time of low tide in the field. Whether this is in fact reflective of an advancing endogenous tidal cycle or is a shifting of the circadian rhythm is not established at this time. The observed suppression of activity under LL is characteristic of both aquatic and terrestrial nocturnal species (Aschoff, 1960).

Semi-lunar reproductive rhythms are well known among intertidal crustaceans, snails, and bivalves (Gifford, 1962; Warner, 1967; Russell-Hunter, Apley and

Hunter, 1972; Wheeler, 1979). The field emergence of larvae on the full moon and the absence of larval release on the new moon therefore poses an intriguing question. Is it reflective of an endogenous lunar rhythm, or is moonlight a key stimulus in orienting the movement of larvae to the surface and into the water column?

Based on the activity of larvae in the laboratory, there appears to be a lunar rhythm of responsiveness to light that is somewhat different from the lunar locomotory rhythms observed. While larvae were active at both full and new moons (*i.e.* on spring tides), they were responsive to light only at full moon. This is consistent with the lack of field larval release during the spring tides of the new moon.

Furthermore, neurophysiological sensitivity to light several hundred times dimmer than moonlight has been recorded for the *Limulus* median ocellus by Lall and Chapman (1973) who obtained responses to levels of illumination as low as $1.4 \times 10^{-10} \mu\text{W}/(\text{cm}^2 \cdot \text{nm})$ at 360 NM. Moonlight intensity of $10^{-3} \mu\text{W}/(\text{cm}^2 \cdot \text{nm})$ has been reported by Kampa (1970). Therefore moonlight is well within the sensitivity range of adult specimens of *Limulus*.

A model to explain the observed pattern of larval release in the field is presented. It is based on the comparison of field release with the activity and light response patterns seen in the laboratory. The following factors are apparently of significance: an endogenous readiness to respond to a dim light source that peaks at the time of full moon and is minimal at the time of new moon; the availability of an appropriate light source in the form of a full moon; a water level reaching the nests only during periods of spring tides; and the suppression of activity by the bright light of day that overrides afternoon spring high tides. This pattern of larval release is supplemented by occasional mass releases caused by storms that coincide with high tides at times other than full moon.

This model seems to be most consistent with the data presently available. In particular, the full/new moon differences in response to light in the laboratory correspond to the lack of field release at new moon. However, whether moonlight or the lack of it is detectable to larvae buried several centimeters in the sand prior to high tide has not been determined. If it is not detectable at the depth of the nests, then some other factor may be instrumental in initiating movement toward the surface.

If inactive larvae are removed from a nest and placed in water, swimming activity is immediately initiated. Increasing interstitial moisture associated with the rising tide might conceivably initiate activity, for instance, with moonlight becoming the dominant stimulus only as the animals approach the surface. The apparent partial movement of larvae toward the surface on the night of new moon in June, 1979, suggests the existence of some stimulus in addition to moonlight. The identification of that stimulus, should it exist, as well as testing of the other components of the proposed model, awaits additional research.

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SUMMARY

The horseshoe crab *Limulus polyphemus* lays its eggs on sandy beaches at the level of the highest high tide in the northeastern Gulf of Mexico, buried approximately 18 cm below the surface. When they hatch, the larvae must move from the buried nest site into the marine environment.

In the field, nests of larvae move to the sand surface and emerge at the spring high tide on the night of full moon. They may also be released by heavy surf associated with storms. No release occurs on the spring high tides associated with new moon.

In the laboratory, larvae are seen to be nocturnally active, both under ambient and DD photoperiods. Activity peaks at times of full and new moons, and larvae are positively phototactic at all lunar phases except new moon.

A model to account for observed field behavior in light of laboratory activity and light responses is presented.

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DEVELOPMENT OF BIOLUMINESCENCE AND OTHER EFFECTOR
RESPONSES IN THE PENNATULID COELENTERATE
RENILLA KÖLLIKERI

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In benthic marine invertebrates with planktonic larval forms, the conversion from larva to adult usually involves significant morphological and physiological changes. The morphological changes can involve destruction and resorption as well as elaboration and reorganization of larval structures, the production of new tissues and structures, and renewed differentiation of adult organ rudiments (see, for example, Bonar, 1978; Cameron and Hinegardner, 1974, 1978). Despite a wealth of information concerning morphological changes which occur at metamorphosis of marine invertebrate larvae, little is known about the physiological changes at this critical time. In particular, little is known about functional changes in the nervous and effector systems during larval maturation and transformation to the adult form.

The ideal preparation for studying changes in larval neuroeffector function is one in which the organization of nervous and effector elements is simple, the systems in question can be easily monitored, the adult neuroeffector systems are well understood, and the larval stages are relatively large and easy to raise through metamorphosis. These conditions are approached in the octocoral *Renilla köllikeri*. The nervous elements of *Renilla* are arranged in a diffuse neural network (Satterlie, Anderson and Case, 1976). This nerve net is through-conducting, that is, an impulse initiated in one area of the net is transmitted throughout the remaining portion without decrement (Anderson and Case, 1975; Satterlie, Anderson and Case, 1976). Effector function in *Renilla*, and other anthozoans, is based on perhaps the simplest integrative mechanism, peripheral frequency dependent facilitation (Pantin, 1935; Parker, 1920; Nicol, 1955a, b) and is therefore relatively predictable. Although larvae of *Renilla* are not large enough for electrophysiological recording, they possess an effector system, the bioluminescent system, which is easily monitored photometrically. Furthermore, in live preparations, the luminescent cells (photocytes) fluoresce when illuminated with the proper exciting wavelength of light. The luminescent system can therefore be monitored both morphologically and physiologically in live, intact specimens. The neuroeffector systems of adult *Renilla* colonies, including the luminescent system, have been examined in some detail (Anderson and Case, 1975). Electrical impulses were recorded from a colonial conduction system which controls bioluminescence, polyp withdrawal and colonial contraction. Semi-autonomous polyp conduction systems are also active (Anderson and Case, 1975).

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Our studies indicate that the development of *Renilla köllikeri* is similar to that of *Renilla reniformis* (Wilson, 1883) and other octocorals (Matthews, 1916; Gohar, 1940a, b; Chia and Crawford, 1973). The eggs of *Renilla* are large and yolky, and are usually spawned in large numbers. A swimming planula settles and metamorphoses into a primary polyp. Secondary polyps bud from the primary polyp to form the colony (see Wilson, 1883).

We have followed the development of *Renilla* from the fertilized egg to the mature primary polyp during three reproductive seasons in order to describe the onset of effector form and function, with particular reference to three activities: ciliary swimming, muscular activities, and bioluminescence. The observations of effector function have been utilized to describe the functional development of the nervous system(s) that underlie effector activation during the various stages of larval life. In another report, these observations will be supplemented by an ultrastructural investigation of the developmental stages.

MATERIALS AND METHODS

Colonies of *Renilla köllikeri* Pfeffer were collected in shallow water off Zuma Beach, California and in the Santa Barbara Channel by divers. Healthy specimens were kept in large aerated aquaria at 18 to 20° C and checked several times daily for spontaneous spawning. Colonies that had been dark conditioned for 1 to 2 days in running seawater aquaria frequently spawned following transfer to lighted battery jars of still sea water. Larvae were reared in 3-liter flasks or beakers of aerated sea water, changed twice daily. Late swimmers (see results for description) were transferred to 250-ml beakers with or without sand. The lecithotrophic eggs permitted development to the primary polyp stage without feeding.

Fluorescence

Living larvae were observed in one of three types of squash preparations. Light and medium squashes involved bridging a coverslip across two other coverslips (No. 1½ thickness) on a slide. For light squashes, the slide was flooded with sea water and for medium squashes, the animal was placed in a small drop. In heavy squashes, which frequently resulted in tissue damage, a coverslip was placed directly on a small drop of sea water containing the specimen. A Zeiss Universal Research Microscope was used with mercury lamp illumination, BG 12 and BG 38 exciting filters, and a 500 nm cut-off barrier filter. Larvae were anesthetized in a 1:1 solution of 0.37 M $MgCl_2$: sea water for photography.

Bioluminescence

Bioluminescence was detected with an EMI 9601B end-window photomultiplier tube operated at -950 V, giving a high signal-to-noise ratio. A Uniblitz 100-2 electric shutter was threaded onto the window end of the tube and operated from outside a dark experimental box. Signals from the photomultiplier tube were amplified with a Keithley 427 current amplifier leading to a 7P1F DC amplifier of a Grass 79D polygraph.

The larvae were tested in laminated plexiglass blocks with 2.5 cm diameter and

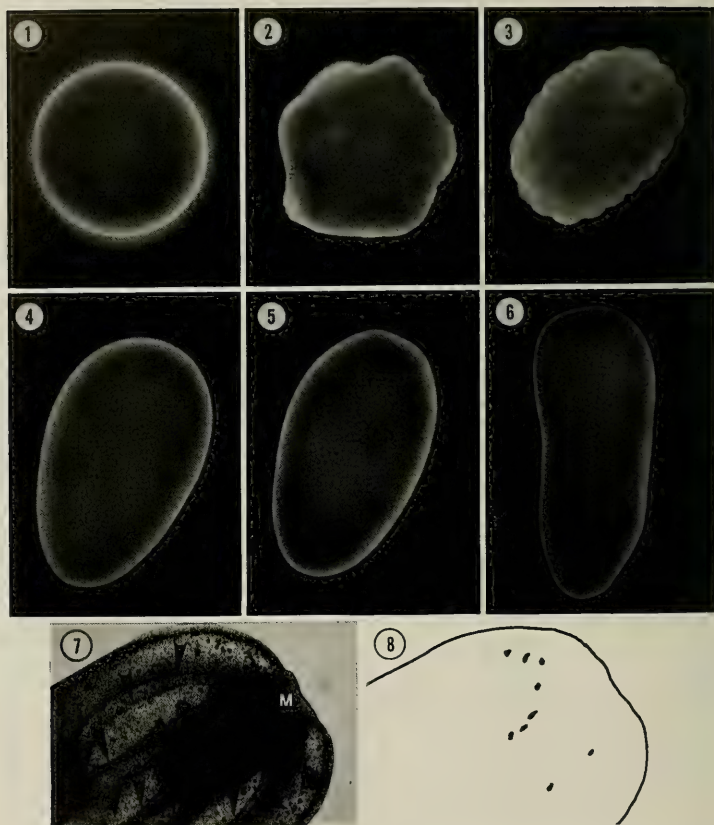


FIGURE 1. Fertilized egg approximately 15 min after release. The ciliary coating is made up of thousands of sperm adhering to the egg surface (verified by E.M.). $\times 76$, light squash.

FIGURE 2. About 8-cell stage. The initial cleavages are irregular and apparently incomplete. At this stage, most of the sperm coating is gone. One hour embryo. $\times 76$, light squash.

FIGURE 3. About 64-cell stage. Three hour embryo. $\times 76$, light squash.

FIGURE 4. Pre-swimmer planula. At this stage, the endoderm is fully formed and the gastric cavity begins to appear. Ciliary activity is weak so the pre-swimmer merely spins in circles on the bottom. $\times 76$, light squash.

FIGURE 5. Early swimmer planula. Ciliary activity is sufficient to lift the planula off the bottom. No muscular activity is evident. $\times 76$, light squash.

1-cm-deep center wells (same diameter as shutter opening) which were positioned to allow a 3-cm specimen-to-pm tube window distance. Chemicals could be introduced via PE 50 intramedic polyethylene tubing penetrating the wall of the chamber. Single larvae were placed in 1 ml of sea water in the center well, which could be centered under the shutter/pm tube by touch. One milliliter of test solution was slowly injected. The test solutions were 0.53 M KCl, 0.1 M CaCl_2 , 3% H_2O_2 , and sea water controls. The electrical test chamber consisted of a similar plexiglass block with a pair of horizontal 180 mesh silver screens (Unique Wire Weaving Co., Hillside, N. J.), leading to a Grass S9 stimulator. Test animals were placed on the bottom screen with enough sea water to cover them but prevent swimming. The upper screen was then pressed down to contact the larva or sea water. Large stimulating voltages were frequently required due to the shunting effect of the sea water. All luminescence experiments were carried out in a dark box within a darkroom at ambient air temperature (20° to 25° C).

RESULTS

Pennatulid colonies have three distinct parts: the peduncle, rachis, and polyps. The basal peduncle is an anchoring device which is inserted into the substratum by peristaltic movements. The rachis, or main tissue mass, supports an array of two types of secondary polyps, the siphonozooids and the autozooids. The rachis and peduncle are derived from the primary polyp, and the colony is formed by budding of the secondary polyps (Wilson, 1883). The rachis of *Renilla köllikeri* is flattened and leaf-shaped. The ventral surface is devoid of polyps and normally lies in the substratum. The dorsal surface bears scattered autozooids, inhalent siphonozooid clusters, and a single exhalent siphonozooid.

Renilla köllikeri colonies are dioecious. Gametes are borne on the autozooid septal filaments. When mature, the eggs are tan in color and approximately 0.3 to 0.4 mm in diameter. In male colonies, sperm are packed in follicles which are the same size as the eggs, but white in color. Both eggs and sperm follicles are covered by a single layer of ciliated endodermal cells until spawning takes place. During the reproductive season, the septal filaments of each autozooid contain more than 20 sperm follicles or eggs at different stages of development.

Spawning

The reproductive season for *Renilla köllikeri* in the Santa Barbara area extends from May to late July or early August, a period centered around the summer solstice. During this period colonies may spawn many times, in small groups or *en masse*. In female colonies, spawning begins with an extreme inflation of the

FIGURE 6. Swimmer planula. The planula is now hollow and the septa begin to form by outgrowth of the endoderm. Swimming is active. Local muscular contractions can be elicited. $\times 76$, light squash.

FIGURES 7-8. Swimmer planula, approximately 10 to 15 hr older than in Figure 6. The same animal was used for both bright field (7) and fluorescence (8, traced from original to show position of dimly fluorescent photocytes) micrographs. The first sign of fluorescence is evident at this time. Note that the anthocodial septa are well formed (Fig. 7, arrows). The mouth (M, Fig. 7) is not yet open. $\times 56$, heavy squash.

rachis, in conjunction with accelerated and pronounced rachidial peristalsis. In a 9.5 cm colony, each peristaltic wave passed across the rachis in 45 to 60 sec, a conduction velocity of 0.16 to 0.21 cm/s (21° C). This is compared to a rate of 0.11 to 0.13 cm/s (23° C) in non-spawning *Renilla*. Two and sometimes three waves are present on the rachis of spawning colonies at all times. During spawning, the autozooids remain extended with the tentacles bent slightly in an aboral direction. In female colonies, the shedding of eggs appears to result from the peristaltic movements. In one instance, three waves were required to squeeze an egg out of the autozoid mouth.

When released, the eggs are oblong, but become spherical in 15 to 30 min (Fig. 1). They are neutrally buoyant, and float at all levels in an aquarium of still water. All released eggs, as well as some dissected from a spawning colony, were already fertilized, indicating that fertilization occurs before release. Sperm follicles are not released intact during spawning. The follicles rupture within the autozooids, and the sperm are released through the autozoid mouths and the exhalant siphonozoid.

TABLE I

Summary of early development of Renilla köllikeri with observations on effector function (25° C). The numbers in parentheses indicate the corresponding text figures.

Hours from spawning	Stage	Ciliary swimming	Muscle activity	Fluorescence	Bioluminescence
0	Fertilized egg (1)	—	—	—	—
½-3	First cleavages (2)	—	—	—	—
1-3	About 64-cell (3)	—	—	—	—
7	Stereoblastula	—	—	—	—
32	Pre-swimmer (4)	Spinning on bottom	—	—	—
38	Early swimmer (5)	Swimming	—	—	—
60	Swimmer (6)	Swimming	Local contractions	—	—
84	Swimmer (7)	Swimming	Conducted contractions	First sign (8)	—
118½	Late swimmer (9)	Swimming, sinking	Conducted contractions	++ (10)	—
131	Settled (12)	Ceases	Conducted contractions	++ (11)	— (20B)
136½	Settled (13)	—	Separate responses	+++ (13) as in adult	First Sign (20C) + (20E)
191	Tentacle buds (14)	—	Separate responses	+++	++ (20F)
274	Pinnules (15)	—	Separate responses	+++ (15, 16)	+++ (20G)
323	Primary polyp	—	Separate responses	+++ (17)	+++ (20G)

Larval development

The early cleavages and larval development of *Renilla köllikeri* are similar to that described by Wilson (1883) for *Renilla reniformis*. The initial cleavages are extremely irregular and variable. Seldom can 2-, 4-, or 8-cell stages be recognized. The first detectable cleavages (*Renilla köllikeri*) occur 20 min to 3 hr after spawning, at which time embryos of 8 to approximately 64 cells are observed (Fig. 3). Prior to this stage the embryos are very irregular in shape (Fig. 2).

Early development progressed more rapidly at 25° C than at lower temperatures tested. Cooler temperatures, down to 6° C, retarded development without apparent harmful effects. A timetable for the development of *Renilla köllikeri* (25° C) is shown in Table I, and all further mention of age will refer to this table and temperature.

Just prior to cleavage, neutral buoyancy is lost, and the embryos settle to the bottom of the aquarium. No ciliary activity is apparent. Subsequent cell divisions give rise to stereoblastulae. Endoderm formation is followed by gradual disappearance of the central, yolky cells. Swimming planulae are formed 38 hr after spawning (Figs. 4, 5). The planulae are barrel-shaped and swim at the air-water inter-

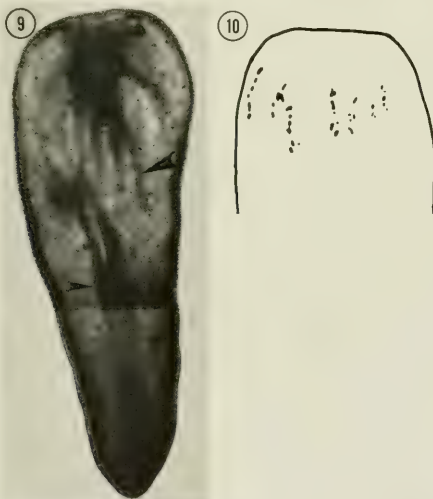


FIGURE 9. Late swimmer planula. The eight anthocodial septa (one indicated, large arrow) are visible, two of which extend to form the peduncular septum (small arrow). $\times 101$, light squash.

FIGURE 10. Late swimmer planula. Tracing of a fluorescence micrograph indicating the position of the dimly fluorescent photocytes, which are arranged in rows along the septa. $\times 47$, medium squash.



face (early swimmers, Table I). During the swimming period (4 to 6 days) the planulae gradually elongate (swimmers, Figs. 6, 7) and undergo considerable cellular differentiation. During this time the peduncular septum, as well as the eight anthocodial septa are formed (Fig. 7). The differentiation of muscle and nerve cells begins early in the planula stage (early swimmers; Satterlie and Case, in preparation).

When 5 days old, the planulae (late swimmers, Fig. 9) begin to sink to the bottom of the aquarium. The larvae settle oral end first on clean glass or in sand (Fig. 12). Settlement is achieved more rapidly when a sand substratum is provided, as opposed to clean glass, the difference being as much as 8 hr. Once attached to the substratum by the oral end, the larvae "roll" to the side and eventually attach by the aboral peduncle. The oral end is then raised. When settled in sand, the larvae move between sand grains and in some cases burrow in approximately a half-centimeter.

Tentacle buds first appear as conical projections on the anthocodium at 8 days (Fig. 14). The tentacles gradually elongate and sprout lateral pinnules (Fig. 15). At 13 days, the juveniles represent structurally complete primary polyps (Figs. 18, 19). The juveniles survived as single polyps for up to 3 weeks in the laboratory, but did not bud secondary autozooids. See Wilson (1883) for an account of colony formation in *Renilla reniformis*.

Development of effector systems

Ciliary swimming. The ectoderm of adult specimens of *Renilla* is not heavily ciliated except for the invaginated pharynx of each polyp. However, for a short time during development, the planulae rely on a dense ectodermal ciliary coat for locomotion. Ciliary activity is continuous, and no ciliary reversals or arrests could be demonstrated. When a planula encountered a solid object, for instance the side of a container, it would continue to swim in a forward direction (aboral end first) until it eventually slid away. The swimming motion produces a rotation about the oral-aboral axis, and in the late swimmer, which maintains a bend in the aboral end, produces a cork-screw-like motion. All ectodermal ciliary activity ceases upon settlement. If larvae are immediately dislodged, however, ciliary activity reappears. This ability is not apparent after the larvae have remained

FIGURE 11. Freshly settled planula. Fluorescence micrograph showing the photocytes arranged along the septa. $\times 56$, heavy squash.

FIGURE 12. Freshly settled planula, longitudinally contracted. $\times 78$, light squash.

FIGURE 13. Settled juvenile. This animal produced a luminescent response similar to that in Figure 20C. The fluorescent cells are as bright as those in the later stages. Note the constriction in the "neck" region. The anthocodium is inflated, and the peduncle deflated, demonstrating the separation in responses in the two areas. $\times 58$, medium squash.

FIGURE 14. Tentacle bud stage. Again note the inflated anthocodium and contracted peduncle. $\times 80$, light squash.

FIGURE 15. Tentacled polyp at stage of pinnule development. Note the photocyte processes. $\times 66$, medium squash.

FIGURE 16. Higher magnification fluorescence micrograph of the photocyte cluster marked by the arrow in Figure 15. $\times 214$, medium squash.

FIGURES 17-18. Anthocodial region of a primary polyp. The photocytes are found in the lateral sides of the tentacle bases. $\times 43$, medium squash.



FIGURE 19. Full view of a primary polyp. Two siphonozooids are visible (arrows). $\times 14$, medium squash.

settled for an hour or more. If the larvae are not allowed to settle, the late swimmer stage is prolonged and further morphological changes, such as tentacle budding, are delayed.

Muscular activity. Obvious muscular activity is lacking until the swimmer stage (60 hr), after elongation is underway. At this point a mechanical or electrical stimulus to any part of the planula produces a local contraction which pulls the stimulated tissue away from the probe. This muscular contraction is not conducted circularly or longitudinally. Within 2 to 5 hr, similar stimuli produce a more widespread circular constriction, and the planulae contract longitudinally to about two thirds of the relaxed length. At this time, the septa are well formed (Fig. 7). By the late swimmer stage, the larvae are capable of bending movements as well as "protective" longitudinal contractions. These conducted muscular events are not observed when the planulae are placed in excess Mg^{++} for 5 min. In the anesthetized state, stimulation only produces local twitches as in the earlier planulae.

At the time of planula attachment, a division between polyp and peduncle reactions is evident. A stimulus to the peduncle produces a conducted contraction, but not always with an accompanying polyp contraction. Similarly, polyp stimulation does not always produce peduncular contractions. As soon as tentacle buds

are apparent, the primary polyp behaves much like the autozooids of a mature colony. An electrical or mechanical stimulus to the polyp causes an inversion of the anthocodium and an overall contraction of the polyp.

Peristaltic movements are not apparent until settlement, when the peduncle begins rapid, strong peristaltic contractions. If an animal is dislodged, the peristaltic contractions cease until resettlement occurs. As with muscular reactions, a definite separation is evident between the anthocodium and the peduncle, and each is capable of separate peristaltic and bending movements.

Development of the bioluminescent system

Fluorescence. The first sign of fluorescence occurs in the swimmer stage approximately 80 hr after spawning. Extremely dim fluorescent cells are evident on the eight anthocodial septa (Figs. 7, 8). The cells are oval to round, 5 to 10 μ m in maximum length, and without noticeable processes. The size of the fluorescent cell bodies does not appear to change much as the larvae grow. Cell processes are first observed as small, stubby projections after settlement has taken place. By the time of tentacle pinnae development, the cells appear morphologically similar to photocytes of mature autozooids (Figs. 15, 16).

The intensity of fluorescence increases until around 140 hr, when it is approximately as intense as in mature autozooid photocytes. No subsequent increase in fluorescence intensity is apparent. If larvae are prevented from settling, fluorescence increases in intensity at the normal rate, but the appearance of the photocyte cytoplasmic processes is delayed. The increase in fluorescence is not affected by the type of settling substratum.

TABLE II

Summary of chemical bioluminescence tests expressed as number of successful trials/number of trials. The data on substrate effects are from a separate experiment using KCl as the stimulant.

Larval stage	KCl	CaCl ₂	H ₂ O ₂
64 Cell stage	0/30	—	0/5
Early swimmer	0/33	—	0/5
Swimmer	0/26	0/1	0/5
Late swimmer	0/34	0/2	0/5
Settled (oral attachment)	1/24	0/2	0/5
Settled (peduncle)	13/16	1/2	3/5
Swimmers (same age as peduncle settled)	0/14	—	—
Tentacle buds	23/23	2/2	4/5
Swimmers (same age as tentacle buds)	8/12	—	—
Mature tentacles	11/11	5/5	5/5

Substratum effect on KCl response—peduncle settled larvae approximately 160 hr from spawn			
Glass settled (no sand)	5/32		
Sand settled	19/20		
Glass settled (sand in container)	5/8		

As the tentacles begin to form, the fluorescent cell groups appear more peripherally in the septa (compare Figs. 8, 10, 11, 13, 15, and 17). When the tentacles are fully formed, the fluorescent cells are found between the tentacle bases (Fig. 17) as in mature autozooids.

Bioluminescence. The bioluminescent capability of *Renilla* was tested with three chemicals known to induce luminescence in adult animals. Potassium chloride, isotonic with sea water, produces transient flashing presumably by randomly depolarizing cells. H_2O_2 produces similar results. Hypotonic $CaCl_2$ was used by Anderson and Cormier (1973) to induce luminescence from lumisomes, isolated subcellular particles which contain all components of the luminescent reaction. In severed autozooids from adult colonies, 0.1 M $CaCl_2$ produces steady glowing for up to 45 min.

No bioluminescence was measurable up to the time of planula settlement (Table II and Fig. 13). Even when 12 late swimmer larvae, ready to settle, were stimulated together with KCl, luminescence was still not recordable. For comparison, *Obelia* sp. hydroids were tested with KCl. In *Obelia* the photocytes, which are fluorescent, are scattered in the stolons and uprights. Thus a piece of tissue can be dissected which contains only one photocyte. The record from a test of such a piece of *Obelia* is shown in Figure 20A, and represents a base of comparison for the *Renilla* luminescence tests.

Freshly settled planulae do not luminesce (Fig. 20B). One to three hours after settlement, the first light is recorded (Fig. 20C). At this stage, the juveniles are attached by the peduncle with the anthocodium raised, and the fluorescence intensity of the photocytes is equal to that of mature autozoid photocytes. In general, no bioluminescence is recorded from animals in which the fluorescence is less intense than in adult autozooids. If larvae are detached prior to rolling over to the peduncular attachment, and prevented from settling, luminescent competence is delayed by up to 24 hr (Table II).

The substratum is important to the development of luminescent ability (Table II). Late swimmers will settle on the sides and bottom of glass beakers if no other substratum is available. Appearance of bioluminescence in glass settled juveniles is delayed just as if settlement is prevented. Occasionally, planulae settled on the glass sides of a sand-substratum container, in which case luminescence developed normally.

The waveform of chemically-induced luminescence is initially irregular (Figs. 20C, D). When approximately 190 hr old, the juveniles exhibit a luminescent waveform similar to that of the adult, namely a rapid rise to peak followed by a slower decay (Figs. 20E-G). At the tentacle bud stage, light emission takes the form of several individual peaks (Fig. 20E) possibly indicating the sequential stimulation of photocytes or photocyte groups. By the tentacled stage, the emission consists of broad flashes up to 4 sec in duration (Fig. 20G). Multiple flashes are frequently encountered, with the initial flash normally being the brightest (Fig. 20F). In general, the intensity of bioluminescence increases with age of the juvenile. The above descriptions of the stages of bioluminescent competence represent the norm of multiple tests. The developmental rates of individuals vary slightly.

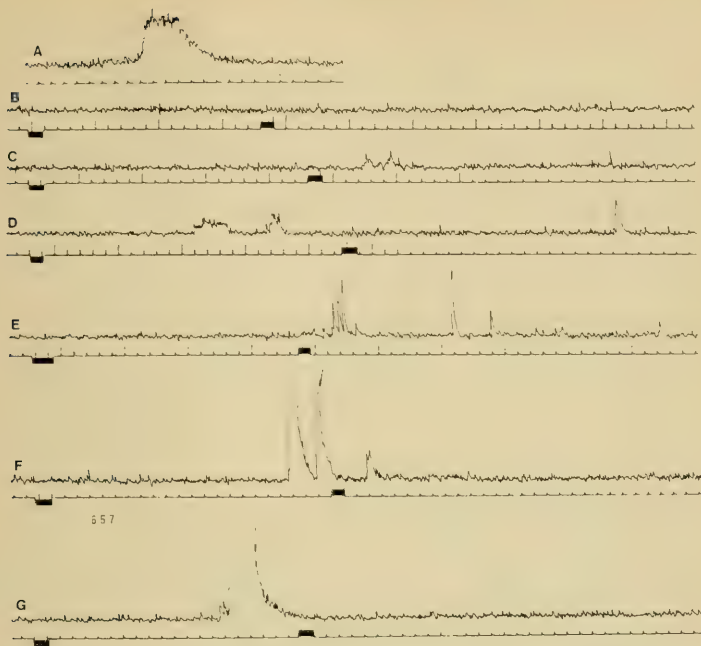


FIGURE 20. Chemically induced bioluminescence in *Renilla* larvae (B-G, see Table II). (A)—KCl induced bioluminescence from a piece of *Obelia* sp. upright containing only one photocyte. The gain is the same for all records. An event marker, superimposed on the time trace (1-second intervals), was used to indicate the beginning (downward mark) and end (upward mark) of the KCl injection. (B)—Newly settled planula; (C)—Settled planula, attached by peduncle; (D)—Juvenile settled for 12 hr; (E)—Tentacle-bud stage; (F)—Pinnule development stage; (G)—Primary polyp. Bioluminescence could not be induced prior to planula settlement.

Settled juveniles luminesce in response to electrical stimulation. Up to the tentacle bud stage (Figs. 21A, B), the voltages required (60 to 80 V/5 ms) result in tissue damage. The responses, however, are similar to those of the KCl tests (Figs. 21A ~ 20C, D; 21B, C ~ 20E; 21D ~ 20F; 21E, F ~ 20G). At the tentacle bud stage and later, the luminescent flashes occur in a 1:1 ratio with stimuli, although in some cases several stimuli are required to initiate the response (Fig. 21C). In subsequent stages, the responses begin with the first or second stimulus (Figs. 21D, E). Stimulus thresholds are on the order of 20 to 40 V/5 ms. Facilitating responses (Fig. 21F), characteristic of adult specimens of *Renilla*, are obtained when the tentacles are fully formed, 320 to 370 hr after spawning

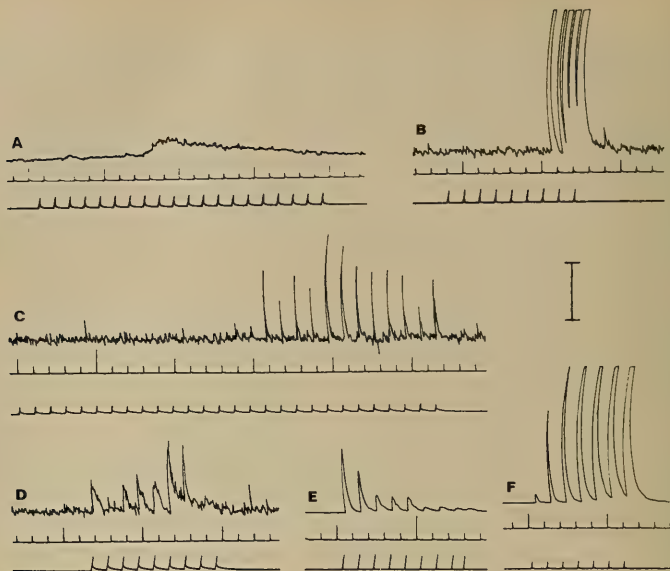


FIGURE 21. Electrically induced bioluminescence in *Renilla* larvae. The top trace represents light output; the center trace, time (1-second intervals); and the bottom trace, stimulus markers (1/s). The scale bar represents the amplitude of the single *Obelia* photocyte luminescent response (Fig. 20A). The gain for traces (A-D) are the same as in Figure 20. The gain for (E) is reduced by 2.5 times, and that for (F) by 5 times. (A)—Settled planula; (B, C)—Tentacle bud stage; (D)—Pinnule development stage; (E, F)—Primary polyp. Bioluminescence responses are initially irregular, eventually following stimuli 1:1. The final stage of maturation of the bioluminescent system is the appearance of facilitating responses (F) similar to those of adult colonies.

(Figs. 18, 19). The stimulus threshold for bioluminescent responses of the mature primary polyp is as low as 5 V/1 ms.

DISCUSSION

The initiating stimuli for spawning in *Renilla köllikeri* are unknown although light seems to be important. Wilson (1883) found that *Renilla reniformis* spawned between 5 AM and 7 AM in the laboratory. Although *Renilla köllikeri* spawned at all times of the day, most spontaneous spawning occurred between 12 noon and 3 PM. By altering the light regime, small spawns could be induced. Released sperm are swept into the female colonies and circulated by the water vascular system, into which the ripe gonads extend. Fertilization is probably internal and perhaps

stimulates female spawning. This behavior could serve to increase the probability of egg fertilization, which would otherwise be jeopardized by the surging currents in which *Renilla* colonies are found (Kastendiek, 1976).

Dispersal of *Renilla* larvae has two phases. The neutral buoyancy of the fertilized eggs allows them to be swept away from the parent colonies. Following loss of neutral buoyancy in the early cleavage stages, the swimming planulae re-enter the water column for 4 to 6 days.

The ciliary swimming behavior of *Renilla* planulae is noteworthy in that a coordinated ciliary reversal or arrest response is lacking. The forward propulsive beat continues regardless of obstacles. A reversal or arrest would require some form of cell-to-cell communication, be it mechanical or bioelectrical. In larvae of many organisms (see Spencer, 1974), as well as in many adult coelenterates (Mackie, 1965, 1975, 1976; Mackie and Passano, 1968; Spencer, 1971, 1975, 1978) impulses are conducted in excitable epithelia. Such a conduction system seems not to be involved in the swimming behavior of *Renilla* planulae unless in the normal swimming metachronism. Intercellular gap junctions could not be found in any of the larval stages of *Renilla* (Satterlie and Case, in preparation).

Muscular activity is first apparent in the swimmer stage, mostly as local responses. The coordinated contractions of late swimmers suggest the presence of a functional conduction system. Both nervous and muscular elements are present by the swimmer stage, and may represent components of this conduction system (Satterlie and Case, in preparation). It is also possible, however, that coordination is due to electrically or mechanically coupled muscle or epithelial cells. Mechanical coupling is counterindicated by the magnesium sensitivity of the muscular responses, which suggests neural control of musculature at this early stage. At least one neural conduction system is apparently present and functional prior to settlement and metamorphosis.

A proper substratum promotes planula settlement. Chia and Crawford (1973) found that sand grain size was not important to the settlement of the pennatulid *Ptilosarcus gurneyi*, but that an organic film on the sand grains was essential to induce settlement. Müller (1973) found that in the hydrozoan *Hydractinia* settlement and metamorphosis is triggered by a lipid produced by marine bacteria. Our findings suggest that a similar situation exists in *Renilla* since settlement of planulae is delayed if clean glass is the only available substratum. As in other octocorals, prevention of attachment prevents or delays metamorphosis. In several xeniid and gorgonian octocorals, delayed settlement increased the number of abnormalities, such as retarded tentacle development (Gohar, 1940b) and axial skeleton formation (von Koch, cited in Gohar, 1940b). Chia and Crawford (1973, 1977) found that planulae of *Ptilosarcus* would continue to swim indefinitely if the proper substratum was not available. Comparing planulae in which settlement was prevented to primary polyps of the same age, they noted significant differences in ultrastructure despite the identical ages of the two groups. Of 9 cell types in the planulae, and 12 cell types in the polyps, only 7 were common (Chia and Crawford, 1977). This reorganization in cellular composition can be attributed to settlement-metamorphosis. Similar settlement-induced changes may parallel the physiological changes which are apparent in the three effector systems

of *Renilla* at the time of peduncle attachment and metamorphosis. The first of these changes is that the ectodermal ciliation becomes inactive and the cilia are apparently resorbed. A second change is that peristaltic movements of the peduncle become intense and a separation can be seen between the muscular activities of peduncle and anthocodium. A separation of conduction systems, into peduncular (future colonial) and anthocodial (future polyp), is therefore suggested. This separation was shown electrophysiologically in adult *Renilla* by Anderson and Case (1975). The metamorphosis-induced changes in neuromuscular organization of *Renilla* larvae probably involve regional differentiation of the planula conduction system since the reactions of the anthocodium and peduncle do not change. Stimuli to either area still produce conducted muscular activity in the stimulated region. During the third change, the juveniles gain the ability to bioluminesce (when about $5\frac{1}{2}$ days old). The green fluorescent protein (see Morin, 1974; Cormier, Hori and Anderson, 1974; Cormier, Lee and Wampler, 1975 for reviews of *Renilla* bioluminescence biochemistry) is produced prior to settlement, and its manufacture is not drastically altered during metamorphosis. The photocytes of the primary polyp are already differentiating prior to settlement, although some morphological changes do occur at this time, such as growth of photocyte processes. The cause of the immediate appearance of bioluminescent competence is not known. The possibility exists that the bioluminescent system was functional prior to settlement, and we were unable to detect the light. However, the multiple animal tests tend to refute this claim. Also, it would be difficult to explain why delays in settlement would also delay the onset of measurable light output.

Colonial bioluminescent responses in adult *Renilla* colonies are controlled by a colonial nerve net (Anderson and Case, 1975; Satterlie, Anderson and Case, 1976). Light emission generally occurs in a 1:1 ratio with stimuli, after the first, and exhibits a facilitation in intensity which is dependent upon interpulse interval (Nicol, 1955a, b). The primary polyps do not exhibit similar responses until tentacle maturation. The sequence of luminescent responses represented in Figures 21C-F could reflect maturation of the neuro-effector hook-ups (within the polyp system), of the conduction systems (colonial and polyp), of the connection between the two conduction systems, or a combination of such developments. Maturation of the polyp neuro-effector system appears to occur between the tentacle bud stage and the tentacled stage, as indicated by the shift from short multiple flashes to "coordinated" broad flashes in the KCl tests. In the latter stage, the presence of a conduction system common to the eight septa, and thus linking the photocyte groups, can be inferred.

The responses of the tentacle-bud stage juveniles are reminiscent of recordings obtained by Nicol (1955a) during bridge experiments on adult *Renilla*. With a small bridge of tissue separating two lobes of the rachis, several stimuli were required before luminous waves were observed in the distal lobe. Interneural facilitation (Pantin, 1935) within the colonial conduction system was proposed as a vehicle for the response. Similar experiments on two other pennatulids, *Stylatula* and *Virgularia*, using electrophysiological recordings of colonial nerve net activity, support this interpretation (Satterlie, Anderson and Case, in prepara-

tion). Immature juvenile conduction systems, or incomplete connections between systems could give rise to local responses. If so, the maturation of the conduction systems or connections, as well as that of the neuro-effector junctions could be represented by a gradual shift to a 1:1 stimulus-to-response ratio with a facilitating intensity output (Figs. 21C-F).

There are two colonial conduction systems in adult specimens of *Renilla*, in addition to semi-autonomous polyp conduction systems (Anderson and Case, 1975). We have demonstrated that at least one conduction system is present in the swimming planula prior to settlement. This conduction system mediates muscle activity of the entire larva (future polyp and colony tissue), and may represent the future colonial nerve net. At the time of settlement and metamorphosis the separation between the polyp and peduncular systems that becomes evident probably involves separation and elaboration of the polyp conduction system(s) and formation of colony-polyp connections. In light of the morphological reorganization that occurs in other pennatulids (*Ptilosarcus*, Chia and Crawford, 1977) at metamorphosis, reorganization of conduction systems in *Renilla* is certainly possible. The final stage in development of the neuro-effector systems, the appearance of facilitating responses, probably involves maturation of the neuro-effector junctions themselves. The role of the second colonial conduction systems of adult *Renilla* colonies is uncertain, and this report does not contribute to its elucidation.

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SUMMARY

1. The development of the pennatulid coelenterate *Renilla köllikeri* was followed from fertilized egg to primary polyp stage, including observations on the development of effector responses such as ciliary swimming, muscular reactions and bioluminescence.

2. Ciliary swimming is first apparent in the early planula, 32 hr after the spawn. Ciliary activity persists until settlement, at approximately 130 hr.

3. Muscular reactions are first evident as local contractions in the swimmer stage (about 60 hr). Conducted contractions can be elicited at 80 hr, suggesting that a functional conduction system is present in the planula larva. At settlement, separate peduncular and anthocodial muscular responses and peristaltic contractions are evident.

4. The future photocytes first fluoresce at about 80 hr, and thereafter fluorescence intensity increases until the time of settlement. The first sign of bioluminescence follows settlement, and is delayed if settlement is prevented. Bioluminescent responses do not exhibit normal facilitation until the primary polyp stage, and responses of the preceding stages may reflect maturation of the

colonial and polyp conduction systems as well as of connections between the two systems.

5. Settlement and metamorphosis are delayed when planulae are reared in containers without sand.

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ON FEEDING MECHANISMS AND CLEARANCE RATES OF MOLLUSCAN VELIGERS

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The teeth of a mammal or the mouthparts of a copepod can tell a knowledgeable biologist much about that animal's feeding habits. Deductions based on the structure of ciliary bands can be at least as useful. This study is part of a larger effort to relate quantitative aspects of ciliary feeding to the morphology of ciliary feeders. Much more extensive biogeographic or taxonomic comparisons of developmental adaptations can be made when feeding capacities of larvae can be predicted from the length of cilia and the lengths of their ciliated bands.

Molluscan veliger larvae possess a lobed velum which produces both feeding and locomotory currents. Those veligers which feed on suspended particles concentrate these particles between two opposed bands of cilia which line the velar edge (Fretter, 1967; Strathmann, Jahn, and Fonseca, 1972; Thompson, 1959; Werner, 1955). The preoral band consists of long compound cilia which produce the swimming and feeding currents. The postoral band consists of shorter cilia which beat towards the preoral band. The combination of the two bands captures and retains particles. Between these bands is a food groove with small cilia which transport particles towards the mouth.

Veligers are tiny feeding machines which convert small eggs into larger juveniles. Parents which produce small eggs can produce large numbers of offspring, but there are costs that limit larval success as larval size is reduced. A reduced feeding capacity is one cost of a decreased larval size. This can take the form of a reduction in the clearance rate (volume of water cleared of particles per unit time) or restriction to a smaller range of particle sizes. In molluscan veliger larvae the clearance rate is likely to be limited by both the length of the velar edge and the length of the preoral cilia (Strathmann, *et al.*, 1972). The size of the particles captured is probably limited by the length of the preoral cilia.

Here we are testing the hypothesis that longer preoral cilia contribute to higher clearance rates. The test consists of comparative observations with high speed microcinematography of movements of the preoral cilia, the postoral cilia and particles captured at the velar edge. Clearance rates are calculated from these measurements. We used three species whose veligers have different lengths of preoral cilia: the oyster *Crassostrea gigas*, the nudibranch *Tritonia diomedea* and the mud snail *Nassarius obsoletus*. Movements of cilia and particles, but no particle captures, were also observed for a veliger of an unidentified species of prosobranch gastropod. Our cinefilms of feeding larvae also have extended previous interpretations of the veliger feeding mechanism.

METHODS

Oyster (*Crassostrea gigas*) gametes were obtained by placing adults in sea water at 30° C until spawning occurred (3 to 4 hrs). The eggs were fertilized at ambient sea water temperatures (12 to 16° C). Larvae of *T. diomedea* and *N. obsoletus* were obtained from eggs laid in laboratory aquaria. The unidentified prosobranch veliger was taken from the plankton in Friday Harbor. All larvae were reared in culture dishes in the laboratory at 12 to 16° C and were fed the green flagellate *Dunaliella tertiolecta*.

Test subjects were starved for 24 hr before being filmed to promote maximum feeding rates during filming. Coverglasses were supported by plasticene feet just low enough to impede larval swimming and high enough not to impede velar cilia in the plane of focus. The larvae were usually filmed with plane of focus perpendicular to the velar edge, as in Figure 4, for velocities of cilia and particles. In some cases larvae were filmed with plane of focus parallel to the velar edge for metachronal wavelength.

Larvae were filmed with Nomarski differential interference optics with 16 or 40× objectives, a high speed cinecamera at 100 or 200 frames per second, and continuous light. A timing light exposed the margin of the film every 0.01 seconds and is accurate to $\pm 1\%$ according to its manufacturer, the Redlake Corporation. Temperatures were maintained at 12° to 13° C with a Cloney cooling stage (Cloney, Schaadt, and Durdeen, 1970) except for some sequences of *C. gigas* filmed at 20° to 22° C.

Particles used for feeding observations were 2- μ m plastic spheres and the flagellates *Dunaliella tertiolecta* and *Monochrysis lutheri* (5 to 10- μ m). We observed only one capture of a plastic sphere so the data on captures apply to the flagellates. High concentrations of particles were used to insure some captures in the plane of focus during high speed filming. Since particles are rapidly removed from suspension by the larvae and also settle in the confined space on a slide, particle concentrations could not be accurately determined. Therefore no cell counts were made.

Tracings of the films were made frame by frame for cilium and particle paths. All velocities were calculated from the tracings. The fraction of particles captured was calculated using all particles passing within reach of the preoral cilia from the beginning to the end of several filmed sequences.

TABLE I

Species	Egg Diameter (μ m)	Shell length at start of feeding (μ m)	Preoral cilium length (μ m)
<i>Crassostrea gigas</i>	45-50	90*	30
<i>Tritonia diomedea</i>	90	145*	40
<i>Nassarius obsoletus</i>	165*	270*	70

* Data from Costello, Davidson, Eggers, Fox, and Henley (1957), Kempf & Willows (1977), Quayle (1969), Scheltema (1967).

RESULTS

We assume that the veligers are actively feeding whenever numerous particles are passing around the food groove to the mouth. When this occurs both the preoral and postoral bands are beating. Occasionally both bands would cease beating but we did not observe the preoral band beating while the postoral band stopped. *Crassostrea gigas*, *Tritonia diomedea*, and *Nassarius obsoletus* are consistent with our impression that the size of veligers and the length of preoral cilia increase with egg size (Table I).

Velocities of cilia and particles

Among species, the velocities of the tips of the preoral cilia in the effective stroke increase with the length of cilium (Fig. 1). It is difficult to see the bases of cilia in many filmed sequences, so radius of arc was estimated by projecting the straight section of a cilium in successive frames back to a point of intersection. For each species the mean estimated radius of arc is greater than the lengths of those cilia which could be measured accurately. The velocities are taken from the maximum movement observed between two successive frames during the effective stroke.

Angular velocities are more convenient for comparisons among species and calculation of clearance rates. The angular velocities plotted in Figure 2 are calculated by dividing the tip velocity by the radius of arc. For cilia which are held

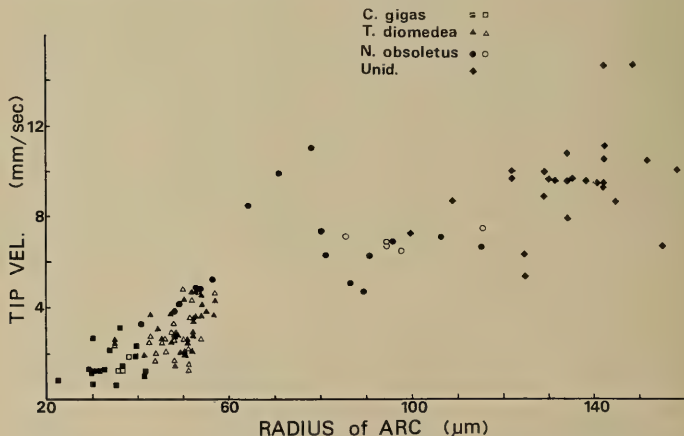


FIGURE 1. Maximum observed tip velocities of preoral cilia during their effective strokes versus radius of arc (estimate of cilium length) for veligers of *Crassostrea gigas*, *Tritonia diomedea*, *Nassarius obsoletus* and an unidentified prosobranch. Open circles are effective strokes associated with particle captures; solid circles with no capture.

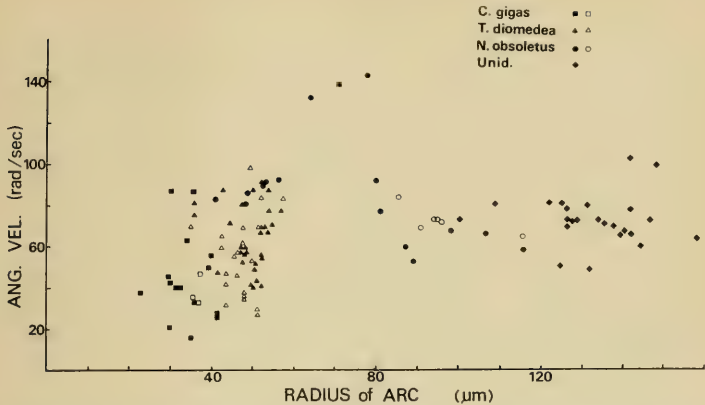


FIGURE 2. Maximum observed angular velocities of preoral cilia during their effective strokes (same larvae as in Figure 1). Open circles are effective strokes associated with particle captures; solid circles with no capture.

straight during the effective stroke, the angular velocity is constant over almost the entire length of the cilium. Angular velocity may increase with cilium length (see below) but the trend is less striking than the increase of tip velocity with the length of the cilium (Fig. 1).

One-way analysis of variance of the angular velocities of the four species indicates significant differences among the species (Table II). A multiple comparison among species indicates that the species fall into three significantly different groups: *C. gigas* with the lowest mean angular velocity, *T. diomedea* in

TABLE II

Comparisons among means for species. H_0 : Means equal; reject if $P < 0.001$

Species	Mean angular velocity (radians/sec)	(n)	Mean ratio of velocities of cilium and particle (C/P)	Range of (C/P)	(n)
<i>Crassostrea gigas</i>	32	(19)	1.6	1.1-2.1	(6)
<i>Tritonia diomedea</i>	54	(51)	2.0	1.1-3.0	(36)
<i>Nassarius obsoletus</i>	70	(25)	1.5	1.0-2.4	(20)
Unidentified	63	(24)	1.5	1.1-1.9	(21)
Reject H_0 (one way Anova) $P < 0.001$			Reject H_0 (Kruskal-Wallis test) $P < 0.001$		

Groups by Student-Newman Keuls multiple comparison (reject H_0 if $P < 0.01$): significantly different groups are (1) *C. gigas*, (2) *T. diomedea*, and (3) *N. obsoletus* and unidentified veliger.

TABLE III

Two sample rank tests for C/P (ratio velocities of cilium and particle) and W (angular velocity of cilium) H_0 : Groups same; reject H_0 if $P < 0.05$. H_0 accepted for all tests (Mann-Whitney U, two tailed).

Species	Comparison	(n ₁ , n ₂)	P
<i>Crassostrea gigas</i>	W at 12° C and 20° C	(24, 23)	>0.20
	W for captures and misses	(3, 3)	>0.20
	C/P for captures and misses	(3, 3)	>0.20
<i>Tritonia diomedea</i>	W for captures and misses	(19, 17)	>0.20
	C/P for captures and misses	(19, 17)	>0.20
<i>Nassarius obsoletus</i>	W for captures and misses	(5, 16)	>0.20
	C/P for captures and misses	(5, 16)	>0.05
	C/P for algae and 2 μ m spheres	(12, 9)	>0.20
Unidentified	C/P for algae and 2 μ m spheres	(16, 5)	>0.20

the middle, and *N. obsoletus* and the unidentified veliger with the highest mean angular velocities (Table II). Preoral cilium length increases along with the angular velocity in these three groups. Thus among species angular velocities of preoral cilia may increase with the lengths of preoral cilia, but more species must be examined to confirm this trend. The observed differences in angular velocities may be associated with the species or the taxonomic order rather than the lengths of the preoral cilia. Also, the mean angular velocity of *N. obsoletus* is greater than for the unidentified prosobranch veliger which has much longer preoral cilia. The implications of the anomalously lower angular velocity in the unidentified veliger are not obvious because this veliger was not observed to catch particles. It is nevertheless clear from these data that the small veligers are not compensating for the shorter length of preoral cilia with increased angular velocities.

Temperature could affect the rate of beat of cilia but we did not observe this. The mean angular velocity of the preoral cilia of *C. gigas* at about 20° C is only slightly greater than at 12 to 13° C, and the difference is not significant (Table III).

The preoral cilia in their effective strokes move one to three times faster than the nearby particles. We could not demonstrate that the ratio of cilium velocity to particle velocity (C/P) varies with the angular velocity of the cilium or the type of particle (Tables III, IV). In *T. diomedea* particle velocities increase and then decrease with the distance from the base of preoral cilium (Fig. 3). The velocities were measured for particles near the middle of the effective stroke of the preoral cilia. Since velocity of the cilium increases from the base to the tip, the ratio of cilium to particle velocity should increase near the tip, but we cannot demonstrate this (Table IV, C/P versus D). Because we cannot show the existence of these possible confounding factors, we have lumped all observations on C/P for each species in a test for differences among species. Differences in C/P among species are highly significant (Table II), but the cause of the difference is not clear. The species with a substantially different ratio of cilium velocity to particle velocity is *T. diomedea*, with a mean ratio of 2 as opposed to about 1.5 for

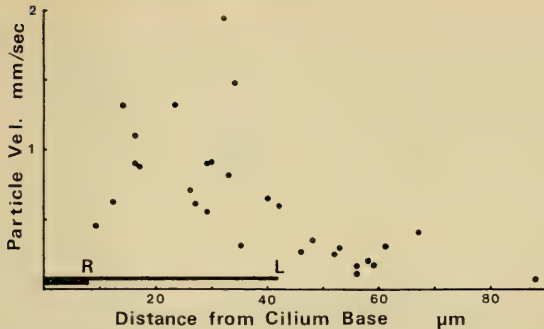


FIGURE 3. Particle velocity versus distance from base of preoral cilium for veliger of *Tritonia diomedea*. L = length of preoral cilium. R = portion blocked by recovery stroke.

the others. The preoral cilia of *T. diomedea* are of intermediate length. We cannot show that the ratio of cilium velocity to particle velocity changes in a regular way with cilium length, cilium speed, or food particle. Therefore, longer preoral cilia, which have angular velocities greater than or equal to those of shorter cilia, move more particles and presumably more water past the preoral band of cilia.

Clearance rate

The volume of water moving through the preoral band can be estimated from the above data. Our estimate combines the angular velocity (W), the ratio of cilium velocity to particle velocity (C/P), the length of the preoral cilium (L), and a correction for the part of the current blocked by the recovery stroke (R). Our reasoning is as follows. The pie-shaped area within an arc of one radian with

TABLE IV

Regression lines for C/P (ratio of velocities of cilium and particles) against W (angular velocity) or D (distance toward base from tip of cilium) H_0 : slope equals 0; reject H_0 if $P < 0.05$ (t -test, two tailed).

Species	Comparison	(n)	P
<i>Crassostrea gigas</i>	C/P versus W	(6)	> 0.50
	C/P versus D	(6)	$0.10 > P > 0.05$
<i>Tritonia diomedea</i>	C/P versus W	(36)	> 0.50
	C/P versus D	(36)	> 0.50
<i>Nassarius obsoletus</i>	C/P versus W	(21)	$0.50 > P > 0.20$
	C/P versus D	(21)	$0.10 > P > 0.05$
Unidentified	C/P versus W	(21)	$0.50 > P > 0.20$
	C/P versus D	(21)	$0.50 > P > 0.20$

TABLE V

Species	L preoral cilium length (μm)	R recovery stroke correction (μm)	$V = (L^2 - R^2)WP/2C$ volume through preoral band ($\mu\text{m}^3/\text{sec } \mu\text{m}$)	F fraction caught out of n particles	(n)	FV clearance rates ($\mu\text{m}^3/\text{sec } \mu\text{m}$)
<i>Crassostrea gigas</i>	30	9	8,200	0.44	(13)	3,600
<i>Tritonia diomedea</i>	40	9	21,000	0.24	(36)	4,900
<i>Nassarius obsoletus</i>	70	9	110,000	0.15	(23)	17,000
Unidentified	100	9	210,000	—	—	—

radius equal to L is $L^2/2$. Subtracting the smaller pie-shaped area blocked by the recovery stroke gives $(L^2 - R^2)/2$. We assume that particle velocity equals water velocity, so this pie-shaped area times the angular velocity times a unit length of the velar edge divided by the ratio of cilium to particle velocities gives the volume of water passing a unit length of the preoral band per unit time as $(L^2 - R^2)WP/2C$. This volume increases with the length of the preoral cilia (Table V).

This estimate includes only the water out to the tips of the cilia and so does not include all the water moved past the velar edge. Three sources of bias may enter this estimate of water flow. First, our measured radius of arc exceeds the length of the cilia, so estimates of angular velocity may be low. Second, we assume a constant ratio of cilium velocity to particle velocity along the cilium, whereas this ratio may increase near the tip. Third, some cilia may have been pushing particles faster than the water. The first underestimates water flow; the second and third overestimate it. For the unidentified prosobranch veliger there is a fourth source of bias. Tips of its preoral cilia are often still bent in the recovery stroke as the base begins the effective stroke, so the effective length of the preoral cilium is less than indicated. The value of R is about the same for all species, as diagrammed in Figure 4, but could not be measured to the nearest μm . Also, R varies around the arc of the effective stroke. Despite these qualifications, the calculation gives an indication of the effectiveness of the cilia in moving water.

To convert this volume per time to an estimate of clearance rate, it is necessary to multiply by the fraction of passing particles which are actually captured. Particles further from the base of the cilia are less likely to be captured, but there is no sharply defined point beyond which no particles are captured and within which all are captured. In Figure 4 velar edges of three species are diagrammed to the same scale. The dashed lines indicate approximate paths of some particles, although the paths vary considerably even at the same distance from the base of the cilia. The films show no captures beyond the outer dashed line. Almost all the particles within the inner dashed line are captured. Particles also tend to travel across the arc described by a cilium. An effective length of cilium is therefore not readily defined.

To obtain the fraction of passing particles which are captured, we recorded the total number of particles passing the preoral band in a filmed sequence of an actively feeding veliger for each species and noted the fraction captured (F, Table V). The sample of captures is small but the sample confirms our impression that the longer preoral cilia are less efficient at removing particles of this size from suspen-

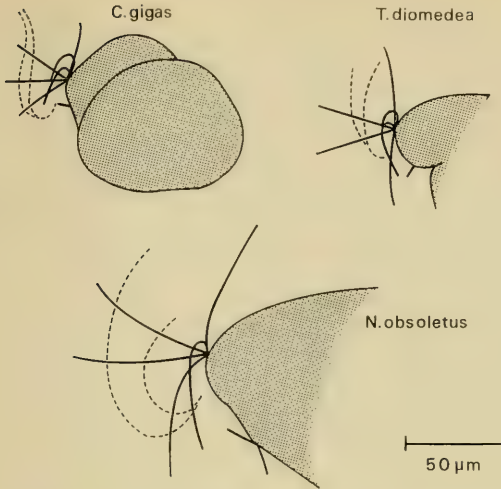


FIGURE 4. Optical section across the velar edge diagrammed for three species to same scale. Preoral and postoral cilia indicated but food groove cilia omitted. Preoral cilium shown in several positions. Dashed lines mark two particle paths (see text).

sion. The clearance rate is estimated by multiplying the volume passing the cilia by the fraction of particles cleared. Though longer cilia are less efficient, there is a definite gain in rate of clearance per unit length of ciliated band with longer preoral cilia.

Mechanisms of concentrating particles

If particles are to be concentrated, they must be moved relative to the water. We have no means of observing water movement in this study, but the observed motion of cilia and particle gives some information on the mechanism by which particles are concentrated. Several combinations of events could occur during concentration: the particles could move faster or slower than the water by either adhering to the cilia or being sieved by two adjacent cilia in their effective strokes. Our observations indicate that more than one mechanism of concentration could be operating.

The simplest hypothesis for concentration is that preoral cilia push adhering particles faster than the water during the effective stroke. In many captures, a preoral cilium overtakes a particle and then moves along next to it. This motion is consistent with the hypothesis that the cilium is pushing an adhering particle faster than the water. Captures occur in which no cilia are observed to touch the particles, but a capturing cilium may have been out of focus.

Particles reverse their direction and move from posterior to anterior into the food groove beneath the preoral cilia in the recovery stroke (Fig. 4). It seems unlikely that the particles are concentrated when they are held or moved against a posterior current here, but we cannot rule out the possibility.

Particles might also be concentrated by a sieve formed by adjacent cilia in their effective strokes. In four captures by the *Tritonia diomedea* veligers two cilia in their effective strokes were separated by an angle of less than 11° . The pair of cilia move next to a particle during part of the effective stroke and could be pushing it faster than the water. Since the gap between the cilia is less than the diameter of the particle, the pair of cilia may be forming a sieve. Because the depth of focus is small, these pairs of cilia may be immediate neighbors. Captures of this sort were not observed with veligers of *Crassostrea gigas* and *Nassarius obsoletus*.

Pairs of cilia separated by a small angle are not visible in most capture sequences. Adjacent preoral cilia are often separated by a large angle in their effective strokes. In such cases the metachronal wavelength rather than the distance between neighboring cilia must set the pore size of the sieve. The preoral cilia of *Nassarius obsoletus* were filmed in a plane perpendicular to the plane of the effective stroke as well as parallel to it. The gap between preoral cilia in the same position of the effective stroke exceeded $20\text{ }\mu\text{m}$ in most instances, whereas these veligers capture particles of a diameter less than $10\text{ }\mu\text{m}$. For veligers of this size the sieve formed by the metachronal wave is only effective for rather large particles, if it is effective at all.

Another hypothesis is that particles are retained by a sieve composed of both the preoral and postoral cilia in the latter part of their effective strokes. Under this hypothesis water in the current generated by the effective strokes would be squeezed out past the cilia of both bands at then end of the effective strokes when the tips of the opposing cilia are close together. We observed a postoral cilium tip meeting a preoral cilium tip in only one of the frames examined. Observed closest approach between preoral and postoral cilium tips in *N. obsoletus* is about $15\text{ }\mu\text{m}$, which is about twice the diameter of the captured particles. The actual distances may be somewhat less, however, because the closest approach can fall in the interval between frames of the film.

The films therefore indicate that the spacing of cilia does not set the minimum size for captured particles and that other mechanisms must be operating either in addition to such sieving or instead of it. The hypothesis of weak adhesion of cilia and particles is consistent with observed movements during captures; the lower frequency of capture of particles nearer cilium tips where differences in velocities are greatest; and the lower efficiency of capture with longer preoral cilia of equal or higher angular velocities. However, direct evidence for adhesion is still lacking.

The role of the postoral cilia in capturing particles is not clear. Occasionally particles enter the food groove past the postoral band but most captured particles do not follow this route. Yet Strathmann *et al.* (1972) found that captures by a serpulid trochophore ceased when the postoral band ceased beating but the preoral band continued beating. Possibly the postoral band is necessary for retaining particles but does not aid in concentrating them. Captured particles reverse their direction of motion where the preoral cilia end their effective stroke. The captured

particles then move anteriorly into the food groove beneath the preoral cilia in their recovery stroke. The recovery strokes of the preoral cilia may be insufficient by themselves to carry the particles into the food groove and keep them there. The current from the postoral cilia may help retain particles during capture and subsequent transport toward the mouth.

DISCUSSION

Our studies of three species of veliger larvae suggest that shorter preoral cilia clear particles from the water current more efficiently than longer preoral cilia but produce lower clearance rates per unit length of velar edge. This lower clearance rate is the result of both shorter cilia and lower angular velocities. However, larvae with longer preoral cilia may capture small particles less efficiently than large particles. Our observations with small flagellates may therefore underestimate the advantages associated with longer preoral cilia. We are not aware of any studies comparing length of preoral cilia to egg size in species of veligers, but our impression, from past casual observations and the three species reared from eggs in this study, is that veligers from larger eggs tend to have longer preoral cilia when they begin feeding. Our tentative conclusion is that veligers from smaller eggs have both a shorter velar edge and a lower clearance per unit length of velar edge, so their maximum clearance rates are lower.

The techniques of this study could be extended to more species and a greater range of sizes of food particles. A broader comparison could establish quantitative relationships between easily measured traits (cilium length and length of velar edge) and feeding capabilities (maximum clearance rate and efficiency of clearance of particles of different sizes). This would permit comparisons of feeding capabilities of larvae in numerous species which would test hypotheses on costs associated with small egg size and geographic shifts in developmental strategies.

Strathmann *et al.* (1972), using Harris' (1961) argument, speculate that with preoral cilia, which are each composed of a bundle of numerous individual cilia, cilium length could vary independently of cilium velocity. There seems to be no physical necessity for higher angular velocities with longer preoral cilia. Observations on more species are therefore needed to establish the trend found with the species studied here.

Rubenstein and Koehl (1977) have categorized the mechanisms by which suspension feeders concentrate food particles. Our observations of veligers indicate direct interception of a particle by a fiber, with the preoral cilium as the fiber. Our best guess from these limited observations is that preoral cilia overtake the particles in the latter part of the effective stroke and weakly adhere to them, pushing them faster than the water. Possibly two preoral cilia sometimes act together as a sieve. The postoral cilia probably help retain particles but do not necessarily play an important role in concentrating particles.

The veligers in this study were maintained at comfortable temperatures during filming, but they were confined on a microscope slide, exposed to bright light, and fed unnaturally high concentrations of particles. They could have reduced their rate of clearance by some means which was not detected. Bayne (1976) calculates a clearance rate of 10,000 to 15,000 $\mu\text{m}^3/\text{sec}$ per μm of velar edge for the veliconcha

of *Mytilus edulis*. This value is based on measurement of the velum and on clearance rates calculated from rates of ingestion by unconfined veligers in a known concentration of *Isochrysis galbana*. Bayne's values fall between those determined here for *T. diomedea* and *N. obsoletus*. The shell length of 250- μ m for the *M. edulis* veliconcha falls between those of early stage *T. diomedea* and *N. obsoletus*, so Bayne's values appear to be in close agreement with our estimates, although the length of preoral cilia of *M. edulis* veligers at this stage is not given. In the worst case, if the preoral cilia of the *M. edulis* veliconcha are about the same as in the early stage *C. gigas* veligers, then our estimates of clearance rates could be one third to one half the maximum rate found by Bayne. In either case, Bayne's result indicates that our values are not far from maximum clearance rates for unconfined veligers.

Strathmann (1971) calculated clearance rates of 5000 to 10,000 $\mu\text{m}^3/\text{sec}$ per μm of ciliated band for echinoderm larvae feeding on *Amphidinium carteri*. This flagellate is somewhat larger than *Monochrysis lutheri*. The cilia of these larvae are shorter than the preoral cilia of *C. gigas* veligers, and the feeding mechanism is different, but the rates are in rough agreement with those determined here. As in Bayne's study, the estimate for the echinoderm larvae is based on ingestion rates of unconfined larvae.

Ratios of preoral cilium velocity to particle velocity in veligers are similar to ratios reported by Sleigh and Aiello (1972), who found ratios of about 1.6, 3.3, and 4.0 near the cilium tips of *Pleurobrachia pileus* comb plates, *Mytilus edulis* gill lateral cilia, and *Stentor polymorphus* membranelles. The plot of particle velocity against distance from cilium base for the veliger of *T. diomedea* is similar to the plots of Sleigh and Aiello for *Stentor* and *Pleurobrachia*, except that the velocities decrease more abruptly beyond the tips of the *T. diomedea* preoral cilia.

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SUMMARY

1. Beat of preoral cilia and particle paths were filmed for veligers of *Crassostrea gigas*, *Tritonia diomedea*, *Nassarius obsoletus* and an unidentified prosobranch. Particle captures were filmed for the three identified species.

2. Clearance rates per unit length of velar edge are estimated from the equation $(L^2 - R^2)WPF/2C$, where L is cilium length, R a correction for recovery stroke, W angular velocity, C/P the ratio of velocities of cilium and particle, and F the fraction of particles captured. The clearance rates are in rough agreement with Bayne's values for veligers of *Mytilus edulis*.

3. In the three identified species, longer preoral cilia clear particles at a higher rate but with less efficiency. Since veligers from larger eggs generally have both longer preoral cilia and a longer velar edge, a larger egg generally produces a veliger with a higher maximum clearance rate when the veliger begins to feed.

4. Angular velocities increase with cilium length in the three identified species of veligers but the larger unidentified species did not continue this trend.

5. Preoral cilia in their effective strokes move 1 to 3 times faster than particles travelling in about the same arc with a mean of about 1.5 times the speed of the particles. In mid effective stroke, the ratio of velocities of cilia and particles is not significantly different for captured and non-captured particles, nor does the ratio vary significantly with angular velocity of cilium. The ratio does vary significantly among species.

6. Particles passing closer to the base of the preoral cilia are more likely to be captured.

7. We hypothesize that suspended particles are concentrated when they are overtaken by preoral cilia in their effective stroke, weakly adhere to the preoral cilia, and are pushed faster than the water. Capture is completed when particles are drawn into the food groove, probably by the action of the recovery stroke of preoral cilia, the current from postoral cilia, or both.

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IRREVERSIBLE NONGENETIC TEMPERATURE ADAPTATION OF OXYGEN UPTAKE IN CLONES OF THE SEA ANEMONE *HALIPLANELLA LUCIAE* (VERRILL)

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For many years physiological differences have been observed among latitudinally separated populations of the same species (Bullock, 1955; Prosser, 1955; Vernberg, 1962). These differences have been attributed to a variety of phenotypic adaptations and also to genetic variation.

One of the earliest examples of physiological variation was the difference in acutely measured bell pulsation rates of the jellyfish *Aurelia aurita* collected from Nova Scotia and Florida (Mayer, 1914), the direction of which agreed with Krogh's (1916) prediction of increased rates in cold-adapted animals compared to warm-adapted animals, when the rates are measured at a common temperature. More recent studies of oxygen uptake as well as motor activity in cnidarians confirmed the existence of distinct responses in latitudinally separated populations within a species (Sassaman and Mangum, 1970; Mangum, Oakes, and Shick, 1972; Shick, 1976).

In many examples of intraspecific variation, latitudinal differences disappear with acclimation to common conditions, indicating their phenotypic origin. In several studies of latitudinally separated populations however, the differences persist despite prolonged exposure to common conditions, which has been interpreted alternatively as genetic divergence and "irreversible nongenetic adaption" (Kinne, 1962). There are numerous examples of variation which is highly likely to have a genetic basis, e.g. interspecific variation (Vernberg, 1962). The possibility of nongenetic but irreversible adaptation within species is less certain.

Evidence of this phenomenon in *Drosophila subobscura* was presented by Smith (1956), who induced variation in heat tolerance among groups of flies raised at different temperatures. The differences, which did not disappear completely upon acclimation to a common temperature, were adaptive in character; flies developing at the higher temperature survived longer at an upper lethal limit. However, selection, operating during development, could conceivably explain these results. Kinne (1962) reported that fish developed from eggs which had been transferred to a different salinity 3 to 6 hr after spawning exhibit lower food conversion efficiencies relative to controls developed from eggs which remained in the spawning salinity. He does not, however, compare food conversion efficiencies of fish acclimated to a common salinity which had developed at different salinities. It is not clear, therefore, whether the observed differences in food conversion efficiencies are reversible upon acclimation to common conditions. Bradley (1978) showed that copepods raised at 20° C are more tolerant of high temperatures than those raised at 10° C. However, these results could have arisen from selection during rearing or to a thermal acclimation that requires more than two days' exposure to a common

temperature to disappear. Schneider (1968) concluded that differences in acclimated oxygen uptake rates among populations of the crab *Rhithropanopeus harrisi* can be reversed only in part by breeding the crabs at a common temperature, but the full report of these data has not appeared.

The mode of reproduction in the actinian *Haliplanella luciae* (Verrill) offers an interesting approach to the question of nongenetic adaptation which is irreversible within a period of prolonged acclimation to common conditions. This species reproduces largely by asexual mechanisms, longitudinal fission being the most common (Shick and Lamb, 1977). The fission process in *Haliplanella luciae* and the effects of temperature and other environmental factors on it have been studied in detail by several authors (Torey and Mery, 1904; Davis, 1919; Uchida, 1932; Miyawaki, 1952; Minasian, 1976). Longitudinal fission can be induced in the laboratory by storing the animal at 10° C or below for 6 to 8 weeks, followed by raising the temperature above 10° C (Sassaman, personal communication). This treatment apparently simulates increasing water temperatures during spring. After fission the progeny regenerate tissues to close the body wall torn in the process. Thus isogenic clones of animals can be raised under different environmental conditions and later compared, virtually eliminating the possibility of genetic variability among the experimental animals.

In the present study, three generations of isogenic anemones were reared at two temperatures, and their rates of oxygen uptake compared after acclimation to common thermal conditions. A few observations were also made on the electrophoretic banding patterns of five enzymes and on the dimensions of the gas exchange surface.

MATERIALS AND METHODS

Collection, maintenance of animals and experimental design

Specimens of *Haliplanella luciae* (Verrill) were collected from the York River estuary (15 to 21‰) at the mouth of Indian Field Creek, Virginia in January 1977 (10° C). Included in this sample were animals of Uchida's (1932) color types 1 and 3, indicating genetic heterogeneity in the population. Assuming all animals to be genetically unique, single individuals were placed in 30-ml plastic beakers filled with York River estuary water. One half of the animals were placed at 28° C and the remainder stored at 18° C. After approximately 2 weeks the animals used at both temperatures had undergone fission, each original individual producing two or more progeny (clonemates). When regeneration was complete, the animals were transferred to 5° C for 6 to 8 weeks. At the end of this period, the clonemates were separated and allowed to undergo fission and subsequent formation of new tissue at different developmental temperatures: one member of each clone was randomly chosen and placed at 18° C and its clonemate was placed at 28° C. After fission and regeneration, the second generation clonemates were separated, stored at 5° C for 6 to 8 weeks and then returned to their developmental temperature, where a third generation was produced. Thus there are four groups of animals according to developmental temperature and the number of generations produced at a given temperature: two consist of animals taken from the field that

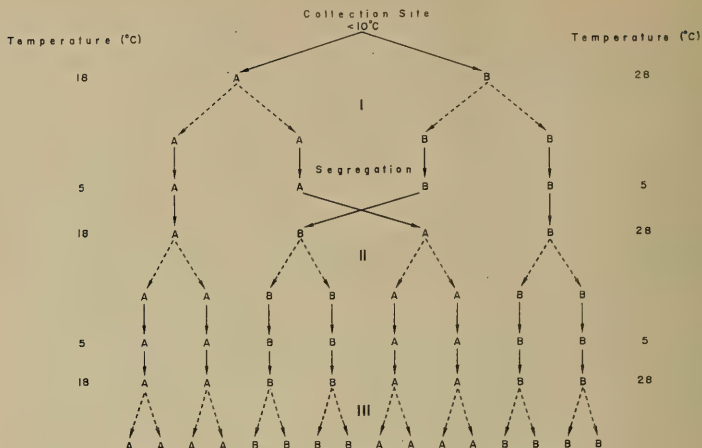


FIGURE 1. Cloning of *H. luciae* at 18° and 28°C . The example shows two animals (A and B) assumed to be genetically unique. Solid arrows indicate transfer from one temperature to another; dotted arrows indicate fission and regeneration. Roman numerals indicate generation.

divided and formed new tissue three times at either 18° or 28°C ; one consists of animals that divided first at 18°C and a second and third time at 28°C , and one of the animals that divided a second and third time at 18°C . Because of the difference in the number of fissions at each developmental temperature (Fig. 1), a control was necessary to permit pooling the data for all clones at a particular developmental temperature. The oxygen uptake rates for animals which divided twice at a developmental temperature were compared with those for animals which had divided three times at that developmental temperature. The important aspect of the experimental design is, however, that each individual has at least one clonemate treated in the same way but given the alternative thermal regime (Fig. 1), and that each of the four experimental groups contains members of the same clones, in the same proportions. Thus the rearing procedures cannot have permitted selection.

Development and growth occurred at the specified temperature in York River estuary water (15 to 21‰, with members of all groups given the same salinity regime). At least 4 days prior to the measurement of oxygen uptake, the salinity was brought to 18‰. Animals were fed an excess of freshly hatched *Artemia* nauplii every other day.

Oxygen uptake measurements

Oxygen uptake ($\dot{V}_{\text{O}_2}$) of the third generation clonemates was measured at the developmental temperature (TD), the same as the acclimation temperature

(TA). These data are designated TD = 18° C = TA and TD = 28° C = TA. Other, but isogenic, individuals were transferred to the alternative temperature, held there for 2 to 4 weeks and oxygen uptake measured. These data are designated TD = 18° C, TA = 28° C, and TD = 28° C, TA = 18° C. All measurements were made in millipore filtered water at 18‰ salinity by adjusting the concentration of natural York River estuary water (15 to 21‰) with distilled water or commercial sea salt (Dayno Corp). Animals were starved for 60 to 84 hr before an experiment, and transferred to the respirometry chambers the night before the measurement to permit attachment to the wall of the chamber. Oxygen depletion was measured with a Yellow Springs Instrument Co. Model 53 Biological Oxygen Monitor, using a high sensitivity (0.0005 in) teflon membrane and a 1 × 10 mm stirring bar. The animals were permitted to adjust to stirring for 1 hr before sealing the chamber and the data were discarded when the tentacles were retracted before completion of the measurement. All data in the P_O₂ interval 110 to 159 mm Hg were analyzed. For each experiment a measurement of the oxygen uptake of the electrode was made using an empty chamber, and this value was subtracted from the experimental rate.

At the end of an experiment animals were stimulated to expel fluid from the gastrovascular cavity, removed from the chambers, lightly blotted and weighed to the nearest tenth of a milligram (Cahn Electrobalance, Model G).

Electrophoresis

Animals were starved for one week and then frozen whole, either singly or in groups from the same clone and treatment group, in two volumes of buffer: 0.05 M tris (titrated to pH 8 with concentrated HCl) and 0.001 M EDTA to which NADP (4 mg/liter) was added. Freezing took place quickly in a bath of dry ice and acetone, and the samples were then stored at -70° C until homogenized. Samples were thawed, homogenized and centrifuged. When necessary the supernatants were diluted as much as 50% with distilled water to obtain 25 μ l which were loaded into slots of horizontal starch gels (13% w/v; Sigma starch).

Gel and electrode buffers for glucose-6-phosphate dehydrogenase (G-6-PDH, E.C. 1.1.1.49) and isocitrate dehydrogenase (IDH, E.C. 1.1.1.42) were modified from Markert and Faulhaber (1965): 0.9 M tris, 0.5 M boric acid, 0.02 M EDTA diluted 1:20 for the gel buffer and 1:6 for the electrodes; in addition, 30 mg and 10 mg NADP were added to the gel and to the cathodal buffer tray, respectively. The gel and electrode buffers for malate dehydrogenase (MDH, E.C. 1.1.1.37) were those described by Nichols and Ruddle (1973). The discontinuous LiOH buffer system of Selander, Hunt, and Yang (1969) was used for phosphoglucose isomerase (PGI, E.C. 5.3.1.9) and hexokinase (HK, E.C. 2.7.1.1). The period of electrophoresis was 3 to 4 hr. After electrophoresis at 350 volts, gels were sliced and stained with slight modifications of the procedures described by Brewer (1970) and Shaw and Prasad (1970). The gels were preserved overnight in 50% ethyl alcohol, blotted and stored in plastic wrap until photographed.

Gas exchange surface area

Animals from the TD = 18° C = TA and the TD = 28° C = TA treatment groups were anesthetized with MgCl₂. Tentacles were counted and the external

surface areas of the body wall and the tentacles were approximated from measurements made at 10 to 25 $\times$, using formulas for the frustum of a cone and for a cylinder, respectively.

Statistical analyses

Mean V_{O_2} values were computed by determining the rate during each time interval of the continuous record of the change in oxygen concentration. Thus, unless specifically stated otherwise, the N value given in the results represents the sum of all data obtained from the members of an experimental group, which are treated as a homogenous population.

The comparison was complicated, however, by the different body sizes of the experimental groups (see below). Two alternative procedures were used to eliminate differences due to body size from the conclusions. First, a regression coefficient of -0.43 was used to correct rates to a common weight of 1.6 mg, a representative value, by covariance analysis. This coefficient was determined by performing a regression analysis on data from the $TD = 18^\circ C = TA$ treatment group ($n = 14$ animals, $r = 0.75$, $P < 0.001$). This relationship is homogenous, showing no evidence of the discontinuity observed by Shick, Brown, Dolliver, and Kayar (1978). Second, the data were grouped by weight classes established from an analysis of frequency distributions, and the comparison made within the classes.

Although the experimental design permits analysis of the data as paired observations, the available procedures for computing Student's t require an equal number of observations in each member of the pair, a condition which was not met. The element of genetic homogeneity was maintained in the experimental design by keeping virtually constant the percent of the total number of observations made on each clone under each combination of developmental and experimental temperatures.

RESULTS

Effect of developmental temperature on body size

At $28^\circ C$ fission occurred more rapidly and more frequently than at $18^\circ C$, resulting in more numerous but smaller progeny (see below). As suggested earlier by a number of investigators (Ray, 1960; Mangum, 1963; Shick, 1972) this finding suggests that the often observed phenomenon of a larger body size in the colder regions of a species range results from latitudinal differences in developmental temperature.

The frequency distribution of weight in each experimental group (Fig. 2) indicates, in general, that body size of the animals is more widely distributed in the $18^\circ C$ developmental group than in the $28^\circ C$ developmental group. Almost 73% of the animals in the $TD = 28^\circ C = TA$ treatment group weigh 1.1 mg or less, and 91% of the animals in the $TD = 28^\circ C$, $TA = 18^\circ C$ experimental group weigh 2.0 mg or less. In contrast, only 29% of the animals in the $TD = 18^\circ C = TA$ group and 17% of those in the $TD = 18^\circ C$, $TA = 28^\circ C$ treatment group weigh 1.1 mg or less. In these groups, reared at low temperature, greater frequencies of animals in the higher weight classes are also evident (Fig. 2). A

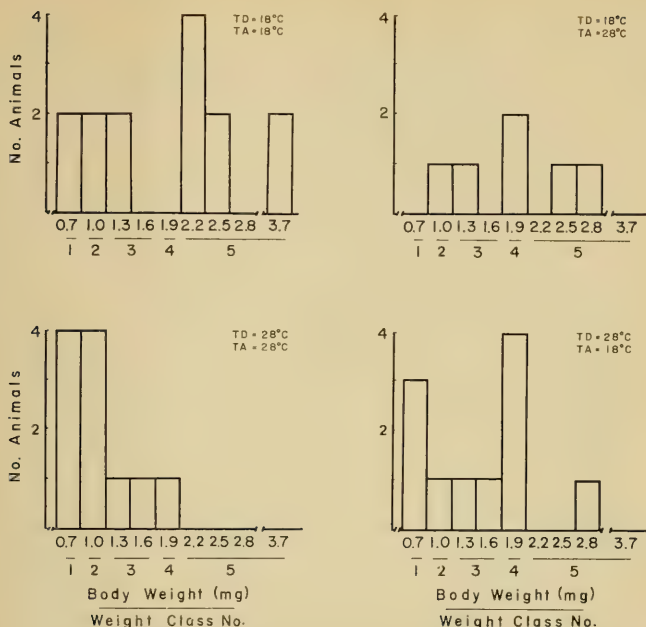


FIGURE 2. Frequency distribution of body weight in treatment groups, designated by developmental (TD) and acclimation (TA) temperature.

comparison of the frequency distributions by weight class of the two developmental temperature groups emphasizes the trends (Fig. 3). The mean body weight of the TD = 28° C = TA and TD = 28° C, TA = 18° C animals is 1.4 mg; the mean weight of the two TD = 18° C groups is 2.0 mg. The median weight class of the two TD = 28° C groups is 0.6 to 0.8 mg, and the corresponding figure for the TD = 18° C groups is 2.1 to 2.3 mg.

Oxygen uptake

Three clones of animals which first divided at 18° C and one clone of animals which first divided at 28° C were used to measure O_2 uptake; all clones produced third generation animals at both developmental temperatures. No significant differences were found between the control groups ($P > 0.85$) indicating that $\dot{V}_{O_2}$ in clones that have produced two generations at a particular developmental temperature is the same as in clones that have produced three generations at that developmental temperature.

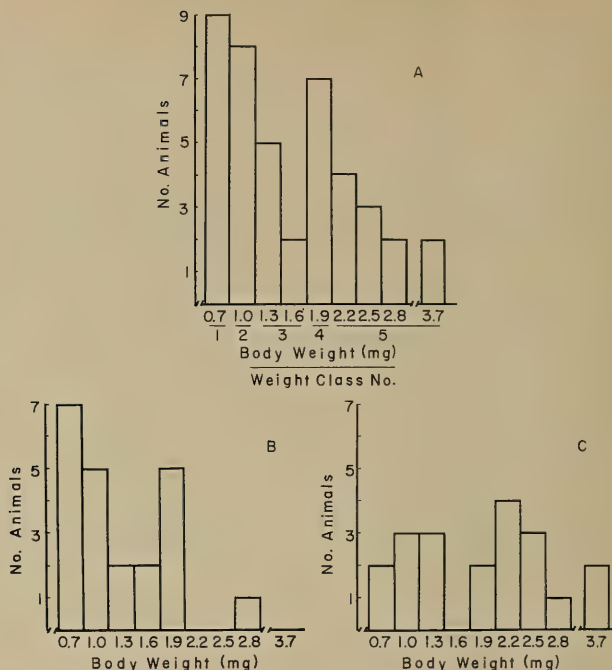


FIGURE 3. Frequency distribution of body weight in (A) all animals used in the oxygen uptake experiment, (B) TD = 28° C developmental groups only, and (C) TD = 18° C developmental groups only.

The mean weight corrected oxygen uptake rates of the two TD = 18° C groups are significantly higher ($P < 0.01$) than those of the two TD = 28° C groups at both acclimation temperatures (Table I). At the test temperature of 18° C the rates in the TD = 18° C groups are 18% higher than those of the TD = 28° C groups, and at 28° C the rates are 19% higher. However, using the raw data for the smaller animals produced at 28° C and the larger ones produced at 18° C, the difference diminished to 4% at the test temperature of 18° C and it is actually reversed at 28° C (-8%). Thus a confounding influence of developmental temperature, its effect on body size, may obscure the direct effect on the acclimated rate of oxygen uptake.

The frequency distribution by weight of all animals used in the oxygen uptake experiments is shown in Figure 3. When the rates are compared by weight class, significant differences between the TD = 18° C and the TD = 28° C groups are

TABLE I

A. Oxygen uptake ($\mu\text{l/g}\cdot\text{hr}$) in isogenic specimens of *Haliplanella luciae* reared for two to three generations at different temperatures. Mean $\pm$ s.e. (N). Probability values from one-way analyses of variance comparing developmental temperature groups at each test (= acclimation) temperature.

Test temperature ($^{\circ}\text{C}$)	Body wt. (mg)	Developmental temperature ($^{\circ}\text{C}$)		P
		18	28	
18	1.9 ± 0.2	243.7 ± 12.0 (111)	235.0 ± 16.2 (72)	n.s.
28	1.3 ± 0.1	487.1 ± 24.1 (36)	524.7 ± 21.6 (54)	n.s.

B. Oxygen uptake ($\mu\text{l/g}\cdot\text{hr}$) corrected to a common body weight (1.6 mg) by covariance. Data analysis as above.

Test temperature ($^{\circ}\text{C}$)	Developmental temperature ($^{\circ}\text{C}$)				P
	18	Q_{10}	28	Q_{10}	
18	257.0 ± 9.4 (111)	1.99	218.7 ± 13.9 (72)	1.96	$P < 0.001$
28	510.3 ± 22.6 (36)		427.8 ± 14.5 (54)		0.0018

found in one of the four possible comparisons at 18°C and in two of three at 28°C (Table II). The trend of higher rates in the TD = 18°C groups is uniformly consistent, but the probability levels rise due to the smaller number of observations (N).

TABLE II

Weight corrected oxygen uptake rates ($\mu\text{l/g}\cdot\text{hr}$), Mean $\pm$ s.e. (N). Probability (P) values from one-way analyses of variance comparing the developmental temperature group rates at each experimental (= acclimation) temperature for each weight class.

Experimental temperature ($^{\circ}\text{C}$)	Weight class		Developmental temperature ($^{\circ}\text{C}$)		P
	No.	Range (mg)	18	28	
18	1	(0.6–0.8)	270.5 ± 46.2 (10)	194.5 ± 33.2 (19)	n.s.
	2	(0.9–1.1)	269.8 ± 38.0 (11)	146.6 ± 41.4 (7)	0.0499
	3	(1.2–1.7)	218.6 ± 22.7 (23)	159.1 ± 21.8 (16)	n.s.
	4	(1.8–2.0)	—	288.5 ± 18.7 (24)	—
	5	(2.1–3.7)	254.7 ± 11.2 (67)	258.8 ± 4.1 (6)	n.s.
28	1	(0.6–0.8)	—	444.0 ± 22.8 (20)	—
	2	(0.9–1.1)	413.3 ± 38.1 (6)	417.4 ± 27.3 (20)	n.s.
	3	(1.2–1.7)	568.9 ± 13.8 (5)	408.3 ± 37.7 (9)	0.003*
	4	(1.8–2.0)	608.9 ± 34.3 (13)	439.9 ± 24.1 (5)	0.01
	5	(2.1–3.7)	427.4 ± 31.6 (12)	—	—

* Bartlett's Test indicates non-homogenous variances for the two developmental groups of this weight class. A Separate Variance Estimate *t*-test was used to make this comparison.

TABLE III

Gas exchange surface area (cm^2/g wet wt.) in each developmental temperature group of *Haliplanella luciae*. N = Number of animals. Weight classes as in Table II.

Developmental temperature ($^{\circ}\text{C}$)	Weight class	N	No. tentacles	Tentacle surface area	
				cm^2/g	% Total body surface area
18	1	2	13	5.6	33.9
	2	2	16	5.0	57.5
	3	3	20	7.6	42.7
28	1	2	18	19.2	73.5
	2	4	30	14.7	72.9
	3	3	29	18.6	78.7

Q_{10} values for both developmental temperature groups (Table II) are similar, and they also approximate the values reported previously over the same temperature range (Sassaman and Mangum, 1970). Thus, no evidence of a change in temperature sensitivity with developmental temperature is seen. No overall pattern of temperature sensitivity is apparent in the data grouped by weight class, thus supporting previous conclusions that it is not correlated with body size (Sassaman and Mangum, 1970). Nor is there a trend in Q_{10} with developmental temperature.

Gas exchange surface

A possible explanation of the irreversible difference between cold and warm-reared anemones is a change in gas exchange surface area. This hypothesis would be supported by an increase in tentacular surface, the primary site of oxygen uptake in epifaunal anemones (Sassaman and Mangum, 1972; Shick *et al.*, 1978) in the $\text{TD} = 18^{\circ}\text{C}$ groups. In fact, measurements of gas exchange surface (Table III) indicate the opposite relationship, suggesting that the difference would be even larger if the gas exchange surface had remained the same.

Surface area is not correlated with body size. Pooling data for all weight classes at each developmental temperature, anemones in the $\text{TD} = 28^{\circ}\text{C}$ groups have more tentacles and a significantly larger (Mann-Whitney U-Test) total surface area available for gas exchange than anemones in the $\text{TD} = 18^{\circ}\text{C}$ groups. Not only are the tentacle numbers greater in the $\text{TD} = 28^{\circ}\text{C}$ groups, but the average surface area per tentacle is greater as well. Moreover, the percent of the total body surface area contributed by the tentacles always exceeds 73 in the $\text{TD} = 28^{\circ}\text{C}$ groups, compared to a maximum of 57.5% in the $\text{TD} = 18^{\circ}\text{C}$ animals (weight class 2).

Electrophoresis

The enzymes examined showed no qualitative differences in banding pattern either among the separate clones of one treatment group or among clonemates of different treatment groups. The results for all of the enzymes tested are similar

and they suggest that the population of *H. luciae* at Indian Field Creek exhibits no variation at these loci. Of the five enzyme systems examined, only IDH was clearly polymorphic, the banding pattern suggesting fixed homozygosity at two loci. However, no qualitative differences in banding pattern were detected, either between clones or within clones. Since these loci differ in latitudinally separated populations and within populations of *H. luciae* (Shick and Lamb, 1977), the uniform pattern could reflect conservative selection pressures on these loci for this particular population. Regardless, there is no evidence that the different alleles were either induced or repressed by the formation of tissues at different temperatures.

DISCUSSION

Differences in aerobic metabolism that cannot be reversed by 2 to 4 weeks acclimation to common conditions clearly result from reproduction, regeneration and the formation of new tissues at different temperatures, when the effects of other variables are eliminated from the data. The direction of the change is generally compensatory, resulting in higher metabolic rates in the animals produced at low temperatures. The magnitude of the change, however, is not great enough to override opposing effects of developmental temperature on body size and gas exchange surface area. Oxygen uptake rates go up at low developmental temperature but down with increasing body size and decreasing tentacular surface, and body size increases and tentacular surface area decreases at low developmental temperature. The net outcome of the concomitant and counteracting trends is little or no difference between the two groups, when body size and gas exchange surface are retained as variables.

The question remains of the origin of the metabolic difference in animals of the same size. One alternative is a direct but irreversible effect of developmental temperature on the metabolic machinery within the cell. The five enzyme systems examined were selected in part for their variability in the species (and in other anemones; Manwell and Baker, 1970), and in part for their relation to aerobic metabolism. Hexokinase, phosphoglucose isomerase and isocitrate dehydrogenase vary in different populations of *H. luciae* (Shick and Lamb, 1977); isocitrate dehydrogenase and malate dehydrogenase catalyze reactions in the tricarboxylic acid cycle and glucose-6-phosphate dehydrogenase regulates a branchpoint of the pentose phosphate pathway. No qualitative changes with developmental temperature or acclimation temperature were found for any of the five enzymes tested. Quantitative data, on various enzyme systems, however, are not available. Robert and Gray (1972) have shown an increase in the specific activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of the blue crab *Callinectes sapidus* during cold exposure, accompanied by no qualitative electrophoretic changes. A thorough test of this possibility, which we regard as a promising hypothesis, would entail an exhaustive investigation of polymorphic enzyme systems that influence the rate of oxidative reactions, directly or indirectly.

The only obvious alternative explanation would be an irreversible effect of developmental temperature on the gas exchange system. Since fluid movements on both sides of the tentacles are generated by cilia, an irreversible difference in oxygen convection would seem to be highly unlikely. An irreversible change in gas

exchange surface was detected, but its effect on oxygen uptake should be the opposite of that observed. Thus a systemic explanation seems highly unlikely.

Regardless of its basis, the present findings clearly demonstrate the reality of irreversible physiological adaptation, and they raise the possibility that the compensatory responses found earlier in geographically separate populations of the species may result from developmental as well as acclimation temperature.

We are grateful to R. J. Hoffmann for the use of his facilities and expertise in performing the electrophoreses, and to D. Reed for his help with data analysis.

SUMMARY

1. Isogenic clones of *H. luciae* were raised at each of two developmental temperatures, 18° and 28° C. Despite prolonged acclimation to common thermal conditions, oxygen uptake rates differ according to the temperature of reproduction, regeneration and development.

2. The effects of developmental temperature, however, are masked by body size differences. Only when this variable is eliminated can the underlying effect of developmental temperature be detected.

3. The irreversible change is not due to an increase in the gas exchange surface area at the primary site of O₂ uptake, the tentacles.

4. No qualitative changes in banding patterns for five enzymes (HK, PGI, IDH, MDH, G-6-PDH) were found.

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